Molecular diagnosis in clinical parasitology: When and why?

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Abstract

Microscopic detection and morphological identification of parasites from clinical specimens are the gold standards for the laboratory diagnosis of parasitic infections. The limitations of such diagnostic assays include insufficient sensitivity and operator dependence. Immunoassays for parasitic antigens are not available for most parasitic infections and have not significantly improved the sensitivity of laboratory detection. Advances in molecular detection by nucleic acid amplification may improve the detection in asymptomatic infections with low parasitic burden. Rapidly accumulating genomic data on parasites allow the design of polymerase chain reaction (PCR) primers directed towards multi-copy gene targets, such as the ribosomal and mitochondrial genes, which further improve the sensitivity. Parasitic cell or its free circulating parasitic DNA can be shed from parasites into blood and excreta which may allow its detection without the whole parasite being present within the portion of clinical sample used for DNA extraction. Multiplex nucleic acid amplification technology allows the simultaneous detection of many parasitic species within a single clinical specimen. In addition to improved sensitivity, nucleic acid amplification with sequencing can help to differentiate different parasitic species at different stages with similar morphology, detect and speciate parasites from fixed histopathological sections and identify anti-parasitic drug resistance. The use of consensus primer and PCR sequencing may even help to identify novel parasitic species. The key limitation of molecular detection is the technological expertise and expense which are usually lacking in the field setting at highly endemic areas. However, such tests can be useful for screening important parasitic infections in asymptomatic patients, donors or recipients coming from endemic areas in the settings of transfusion service or tertiary institutions with transplantation service. Such tests can also be used for monitoring these recipients or highly immunosuppressed patients, so that early preemptive treatment can be given for reactivated parasitic infections while the parasitic burden is still low.

Keywords: Parasites, molecular diagnosis, nucleic acid amplification

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Introduction

Diagnostic clinical parasitology is traditionally a highly visual subject. Different genera and species of parasites are differentiated by differences in their morphology. The standard means of identification involves examination of stained specimens (using temporary or permanent stains) under light microscopy, either with routine parasitological stains or in histopathology sections. Electron microscopy is sometimes necessary to distinguish ultrastructural differences that are essential for species identification. This is especially important in very small protozoa such as the microsporidia for which light microscopy does not offer adequate resolution.

While the microscopic examination for parasites remains the cornerstone of everyday diagnostic parasitology supplemented by immunoassays, the use of molecular assays has gained increasing popularity in the past two decades. With the decline in the prevalence of autochthonous parasitic diseases in many developed countries, expertise in diagnostic parasitology among the new generation of laboratory staff in smaller laboratories may be lacking. This is aggravated by a relatively low number of requests received, lack of diversity in the parasites encountered, and unfamiliarity with unusual or exotic parasites seen in the returned travellers and the immunocompromised hosts.[1,2] Similarly, in the ''field'' setting of developing countries, expert microscopists may not be available, leading to suboptimal diagnosis of parasitic infections.[3] Molecular diagnostics may bridge some of these shortcomings of conventional microscopy. Nevertheless, molecular biology cannot be the solution to all problems in diagnostic

parasitology, and the use and choice of such tests should take into account their limitations and the needs of the laboratory.

Excellent reviews have been published on the use of molecular methods for diagnosing parasitic infections.[4–6] The technical details of various methodologies will not be repeated here. In this article, we explore the situations where molecular assays may be considered as valuable additions to the routine diagnostic service in a clinical laboratory.

Methodological considerations

The most widely used molecular methods for diagnostic parasitology are nucleic acid amplification tests (NAATs). Polymerase chain reaction (PCR) is certainly the most widely adopted technique, including its many variations such as conventional PCR, real-time PCR, multiplex PCR, PCR-ELISA, and so on. Sequencing of the amplicons is sometimes needed for species identification. The targets for NAAT are highly varied. The 18 S rRNA sequences and internal transcribed spacer (ITS) regions are commonly used.[4] Other gene targets may include the cytochrome c oxidase subunit 1 (*cox1*) gene, NADH dehydrogenase, or antigens specific to the genus or species to be detected.[4–7] Among all the gene targets used for molecular diagnosis, the mitochondrial genes are particularly useful because there are often multiple mitochondria present in each cell. Using *Caenorhabditis elegans* as a model nematode, each cell contains 40–70 copies of mitochondrial DNA and 55 copies of 18 S rDNA genes.[8,9] In *Plasmodium falciparum*, there are about 20 copies of mitochondrial DNA and 7 copies of 18 S rDNA genes.[10,11] The use of these multi-copy genes as targets for nucleic acid amplification will increase the sensitivity of the tests. However, not all pathogenic parasites have multiple mitochondria as in other eukaryotes. Members of Kinetoplastida (such as the trypanosomes) and Apicomplexa have only one single mitochondrion per cell. The mitochondrion-like organelle (mitosome) of *Cryptosporidium parvum*, for example, even lacks a mitochondrial genome.[12–14] Another important considerations in the choice of target sequence(s) is the availability of the gene sequence data for a broad range of parasites (Table 1). In contrast to most bacteria and viruses, sequence data of some gene targets of parasites are incomplete. Hence, more than one target may have to be used for accurate species identification, especially for less commonly encountered species.

The sensitivity of NAAT is well recognized. One of the limitations to its broader application in smaller laboratories and developing countries is the need for expensive instruments and reagents. The clinical and public health significance of many parasitic diseases are highest in resource-limited countries where the need for sensitive and specific diagnostic facilities is highest. For this reason, various isothermal amplification techniques have been developed. In particular, loop-mediated isothermal amplification (LAMP) appears to be a promising technique for resource-limited settings. LAMP has been successfully applied for the detection of a variety of parasites from clinical specimens, including blood, urine, faeces, and muscle.[15–21] The use of LAMP for the sensitive and accurate detection of *Plasmodium*, for example, has been evaluated in field settings. It appears to be a suitable technology for this purpose and might have a role as a point-of-care test.[22–26]

Improved sensitivity for diagnosis

The higher sensitivity of NAAT as compared to conventional microscopy in the detection of parasites is best quantified in the diagnosis of malaria. The detection limit of a thick blood smear is about 50 parasites per microlitre of blood when examined by experienced microscopists.[27] In recent years, various immunochromatographic assays (based on *Plasmodium*- and *P. falciparum*-specific lactate dehydrogenase, *Plasmodium* aldolase, and histidine-rich proteins) enabled more rapid diagnosis of malaria independent of microscopy. These rapid diagnostic tests, however, cannot differentiate all *Plasmodium* species, and the test remains positive despite successful treatment. More importantly, their sensitivities drop significantly at low levels of parasitaemia.[28] NAAT can readily achieve a detection limit of less than five parasites per microlitre of blood.[29] A positive PCR reaction, however, has to be interpreted with the clinical setting because parasite DNA in the blood may persist despite successful chemotherapy.[30] This high level of sensitivity not only allows detection of low-level parasitaemia for clinical diagnosis, but is potentially useful in screening of blood products by blood banks to prevent transfusion-transmitted malaria.[31] With proper validation, a NAAT-based strategy may also apply to screening of other blood-borne parasites such as *Babesia* spp. and *Trypanosoma cruzi*.[32,33]

The sensitivity of NAAT assays have been successfully used in the diagnosis and monitoring of other blood and tissue parasitic infections in which the parasite load in the clinical specimen could sometimes be low. Examples include human African trypanosomiasis, Chagas disease, visceral leishmaniasis, babesiosis, filariases, and eosinophilic meningitis due to *Angiostrongylus cantonensis*.[34–42] Although the high sensitivity is one of the biggest advantages of NAAT assays, one should bear in mind that the low parasitaemia level seen in some infections (such as the chronic phase of Chagas disease, human African trypanosomiasis, and visceral leishmaniasis) may cause false negativity in these assays. The effect of a low parasite load is compounded by the so-called ''sample volume effect'', in which a very small quantity of clinical sample is usually used for nucleic acid extraction. With the use of quantitative PCR, the parasitic load in blood can be serially monitored. This is especially important in diseases such as visceral leishmaniasis in HIV-infected patients who may have suboptimal response to chemotherapy and are prone to relapse of the infection.[43,44] The parasitological response of difficult-to-treat parasitic infections can likewise be monitored by quantitative PCR, as in the case of treatment of chronic Chagas disease.[45,46]

The diagnosis of congenital parasitic infections, such as toxoplasmosis and Chagas diseases is sometimes difficult because of the problems associated with conventional

Table 1 Consensus primers for molecular diagnosis of common helminthes in human

Class	Order, family	Genus and species	Target genes	Sequences	Target length (bp)	References
Trematoda	Schistosomatidae	*Schistosoma haematobium, S. mansoni, S. japonicum, S. mekongi, S. intercalatum, S. sinensium, S. malayensis*	18 S rRNA	F–5′ CCTATCAATTTGTTGGTAGGTGATT 3′	210	
				R–5′ TCGAATTACGGAGCCTCAAT 3′		
	Opisthorchiidae, Fasciolidae	*Clonorchis sinensis, Opisthorchis viverrini, O. felineus, Fasciola hepatica, F. gigantica*	18 S rRNA	F–5′ GGAACGGGTGGATTTATTA 3′	138	[192]
				R–5′ AGGGCAGACATTTGAAAGA 3′		
			NADH dehydrogenase subunit 2	F–5′ TTAGAGGAGTTGGTGTCCCC 3′	612	[193]
				R–5′ AGCGTCACTGAACCACACCCAC 3′		
	Troglotrematidae	*Paragonimus westermani, P. mexicana, P. skrjabini*	*cox1* (cytochrome oxidase subunit 1)	F–5′ TCTYTGTTTGGBTATTATGGNTT 3′	272	
				R–5′ ATACCAGTTACACCNCCDATNGT 3′		
	Fasciolidae, Heterophyidae, Paramphistomidae	*Fasciolopsis buski, Metagonimus yokagawai, Wastsonius watsoni, Gastrodiscoides hominis*	ITS	F–5′ TATCACGACGCCCAAAAAGTC 3′	325	
				R–5′ AWTCACGTCTGAACCGAGGTCA 3′		
Nematoda	Filarioidea	*Dirofilaria hongkongensis, D. repens, D. immitis, Loa Loa, Brugia malayi, Masonella perforata, M. atelensis, M. ozzardi, Onchocerca volvulus, Wuchereria bancrofti*	18 S-ITS1-5.8 S	F–5′ CACATCATCATCATCATTATTACTA 3′	461	
				R–5′ TAGCTGCGTTCTTCATCGA 3′		
			cox1	F–5′ GCTTTRTCTTTTTGGKTTACTTTT 3′	350	
				R–5′ TAGTRTCATAAAAAGAAGTATTAAA 3′		
	Rhabditida	*Strongyloides stercoralis*	18 S rRNA	F–5′ ACCGTAAACTATGCCTACTAGA 3′	244	
				R–5′ AACCACTAAATCATGAAAGAGCTA 3′		
	Strongylida	*Necator americanus, Ancylostoma duodenale, A. brazilense, A. caninum*	5.8 S–18 S	F–5′ GATGAGCATTGCWTGAATGCCG	380–485	
				R–5′ GCAAGTRCCGTTCGACAAACAG		
	Thelazioidea	*Thelazia callipaeda, T. gulosa, T. lacrymalis, T. rhodesi, T. skrjabini*	ITS	F–5′ CGTAACAAGGTTTCCGTAGG 3′	900	
				R–5′ GCTGCGTTCTTCATCGAC 3′		
	Ascaridida	*Ascaris lumbricoides*	18 S rRNA	F–5′ CCATGCATGTCTAAGTTCAA 3′	147	[194,195]
				R–5′ CARAAAWTCGGAGCTTTGGT 3′		

(continued)

Table 1 Continued

Class	Order, family	Genus and species	Target genes	Sequences	Target length (bp)	References
			cox1	F–5′ GTTAGGTTACCGTCTAGTAAGG 3′	142	194,195
				R–5′ CACTCAAAAAGGCCAAAGCACC 3′		
	Toxocaridae	*Toxocara canis, T. cati, T. malaysiensis, T. leonina*	18 S rRNA	F–5′ TTCTAGAGCTAATACATGCACCAA 3′	164	
				R–5′ AGTTGATAAGGCAGACACTTGATA 3′		
			ITS2	F–5′ AGTATGATGGGCGCGCCAAT 3′	380	
				R–5′ TTAGTTTCTTTTCCTCCGCT 3′		
	Oxyurida	*Enterobius vermicularis*	ITS	F–5′ CGCCCTAGTTCTGACCGTAA 3′	595	196
				R–5′ GGAGGATTTTCAGGGGGTTA 3′		
	Adenophorea	*Trichinella spiralis, T. nativa, T. britovi, T. murrelli, T. nelsoni, T. pseudospiralis, T. papuae, T. zimbabwensis*	5 S	F–5′ GCGAATTCTTGGATCGGAGACGGCCTG 3′	210	197
				R–5′GCTCTAGACGAGATGTCGTGCTTTCAACG 3′		
	Trichuridae	*Trichuris trichiura*	ITS	F–5′ CTACGTCCCTGCCCTTTGTA 3′	367	
				R–5′ CTCTTCAGACCTGGAACTGTAA 3′		
	Metastrongylidae	*Angiostrongylus vasorum, A. dujardini, A. malaysiensis, A. costaricensis, A. cantonensis*	18 S rRNA	F–5′ CAGATGTSATTTATTCGGAAAA 3′	189	
				R–5′ CGGATTATCGGCGAACAA 3′		
			66 kDa native protein	F–5’ CTCGGCTTAATCTTTGCGAC 3’	232	198
				R–5’ AACGAGCGGCAGTAGAAAAA 3’		
Cestoda	Cyclophyllidea	*Echinococcus granulosus, E. equinus, E. canadensis, E. felidis, E. ortleppi, E. multilocularis, E. oligarthrus, E. vogeli, E. shiquicus*	*cox1*	F-5′ TTGAATTTGCCACGTTTGAATGC 3′	792	199
				R–5′ GAACCTAACGACATAACATAATGA 3′		
	Taeniidae	*Taenia saginata, T. asiatica, T. solium*	18 S rRNA	F–5′ AGTCGGCGACGGGTCCTT 3′	145	
				R–5′ TGCCTCCCTTGGAAGTGGTA 3′		
			cox1	F–5′ TTTAGTTTATTAATTCGTGTTAAT 3;	397	
				R–5′ AGCTAAATGTAAAGAAAACATTA 3′		
	Pseudophyllidea	*Spirometra decipiens, S. folium, S. erinaceieuropaei, S. proliferum*	ITS	F–5′ CGCAGCCAACTGTGTGAATT 3′	603	
				R–5′ ACTGATCCGAGGTCAGGCAA 3′		
			cox1	F–5′ GTGTGTTTAGGTAGNGTNGTTT 3′	258	
				R–5′ TTTTATCCAAYACACAAGCAGAA 3′		

serological diagnosis, presence of low levels of parasites, and sometimes the need for serial monitoring of serology and clinical manifestations before the diagnosis can be fully excluded. The high sensitivity of NAAT assays makes them a useful adjunct to the prenatal and neonatal diagnosis of infections.[47–51] Suitable specimens include maternal blood, placenta, amniotic fluid (not for *T. cruzi*), and neonate's blood.[52]

A recent breakthrough in the molecular diagnosis of parasitic infection is the ability to detect cell-free DNA of *Schistosoma* in serum/plasma and other body fluids of patients.[53–55] As in the case of most helminthiases, the definitive diagnosis in the prepatent period is difficult. Although serological tests for antigens or antibodies are available for some helminths, these are not widely available and their utility is sometimes limited by cross-reactivity between different parasites. However, the detection of circulating cell-free DNA is probably most sensitive for intravascular helminths such as *Schistosoma* in which cells of parasitic origin are constantly shed into the circulation. The usefulness of this approach to other tissue helminths, either larval or adult stages, and those with a migratory larval stage, remains to be confirmed. For intestinal helminths, microscopic diagnosis based on the detection of ova is sometimes limited by the irregular shedding of eggs from the parasites, thereby necessitating the examination of multiple stool samples for definitive diagnosis. False-negative microscopy results may also be encountered in light infections. PCR has been shown to be comparable or superior to microscopic examination of multiple faecal samples in terms of sensitivity in the diagnosis of intestinal helminthiases.[56–59] The higher sensitivity may be attributed to the shedding of parasitic DNA from parasitic components such as cuticular cells rather than actual presence of a parasite within the portion of clinical sample used for DNA extraction. In Hong Kong, an endemic area for clonorchiasis, our study also confirmed the higher sensitivity of PCR for the detection of *Clonorchis sinensis* in both the stool and bile (unpublished data). In 100 stool samples from a regional hospital in Hong Kong, PCR detected *C. sinensis* in 11 specimens, while microscopy only detected 9. Similarly, PCR detected 14 cases of clonorchiasis in 96 bile samples, only 6 of these were detected by microscopy.

Precise species differentiation

Exact parasitic speciation is sometimes not essential for therapeutic purposes. For example, different species of hookworms (*Necator americanus* and *Ancylostoma duodenale*) or tapeworms (taeniasis due to *Taenia solium* and *Taenia saginata*), while their ova are indistinguishable morphologically, are treated with the same antiparasitic regimens. However, parasitologically and epidemiologically speaking, it is always desirable to have the exact identification of the species, as some of the morphologically similar parasites may have different pathogenicity (commensals vs. pathogens, and hence the need for antiparasitic treatment) and may require antiparasitic treatment (mixed infection due to different species), or they may have different intermediate hosts which will impact on control programmes. Precise speciation based on morphology is difficult or impossible in a number of situations.

Cysts or trophozoites of protozoa having similar or indistinguishable morphology

Although the morphology of certain protozoa species or subspecies is identical, they can readily be distinguished based on their unique geographical distributions. *Trypanosoma brucei* is one such example, with *T. brucei gambiense* restricted to central and western Africa, while *T. brucei rhodesiense* is found in eastern and southern Africa. Species differentiation also has clinical significance in that *T. brucei rhodesiense* typically has a rapid disease progression.[60] However, both subspecies may occur in Uganda and molecular methods, such as multiplex PCR, offer a rapid way to distinguish the two subspecies and from other zoonotic species of trypanosomes without having to perform animal inoculation studies or indirect fluorescent antibody test.[61]

The presence of two morphologically similar species, the pathogenic *Entamoeba histolytica* and the commensal *E. dispar,* was postulated since 1925 and subsequently confirmed by clinical, epidemiological, serological, and molecular studies.[62] The differentiation of the two species used to be tedious, requiring culture and isoenzyme electrophoresis.[63] In recent years, the availability of stool antigen detection assays by immunochromatographic or enzyme immunoassay formats based on *E. histolytica*-specific antigens (such as Gal/GalNAc-specific lectin and serine-rich antigen) has simplified the diagnostic process.[63] Although such antigen detection kits are simple, quick, and relatively inexpensive to perform, some kits may perform poorly in clinical settings.[64] A combination of microscopy and a carefully chosen antigen detection assay should satisfy the needs of most routine diagnostic laboratories. NAAT generally possesses higher sensitivity as compared to microscopy and antigen detection, but the difficulties in extracting sufficient nucleic acids from stool samples have to be considered.[63] A variety of techniques such as single-target PCR, multiplex and nested PCR, PCR with restriction enzyme analysis, real-time PCR, and sequencing have been developed for the detection of different species of *Entamoeba* in clinical specimens, including liver abscesses.[63,65]

Entamoeba moshkovskii is a relatively new member of the *Entamoeba histolytica/dispar* complex, which shares the same morphological appearances. Initially thought to be a non-pathogenic species, *E. moshkovskii* has now been associated with diarrhoeal disease in different parts of the world, although its true pathogenicity requires further confirmation.[66] The ability of *E. histolytica*-specific antigen assays to detect *E. moshkovskii* is uncertain; some studies showed that certain ELISA assays are unable to detect *E. moshkovskii*.[67] Currently, molecular methods remain the most definitive methods in the diagnosis of *E. moshkovskii* infections.[68,69]

Clinically suspected leishmaniasis is traditionally confirmed by histopathology, culture of the parasite, or serology. These methods, nevertheless, are either insensitive or cumbersome. The availability of

immunochromatographic antibody tests using K39 or recombinant K39 antigens (commercially available) and antigen detection assays may improve the diagnosis of visceral leishmaniasis.[70] While the species of *Leishmania* in a patient can be suspected by the clinical manifestations and geographical location, multiple species may coexist in the same locality. Isoenzyme analysis was previously used for identification of individual species. Currently, NAAT assays (chiefly by PCR) are much more sensitive than conventional diagnostic tests, and enable accurate speciation of the parasites. Precise species identification aids the development of the most appropriate local control strategies (for example, distinguishing between anthroponotic versus zoonotic forms of *Leishmania* spp.), understanding the epidemiology, and choosing the most suitable antiparasitic regimens. Species identification can be achieved by real-time PCR, PCR-restriction fragment length polymorphism (RFLP), sequencing, or hybridization probes. These techniques have been successfully used for both New World and Old World species, as well as in different clinical forms of leishmaniasis.[71–78] Visceral leishmaniasis and HIV/AIDS are particularly formidable clinical problem. While HIV infection promotes the intracellular uptake and survival of *Leishmania* in macrophages, *Leishmania* infection also enhances the replication of HIV in the host cells.[79] Concurrent HIV infection is uniformly associated with a substantially higher mortality due to visceral leishmaniasis, higher treatment failure and relapse rates, as well as poorer responses to antiparasitic treatment.[79] PCR has become one of the tests of choice for the diagnosis of visceral leishmaniasis in AIDS patients.[79,80] The ability to quantify the parasitic load is especially useful in the management of HIV-associated visceral leishmaniasis, especially in the detection of relapses. Relapses of visceral leishmaniasis in AIDS patients occur in 15–57% of the cases, most commonly in patients with $CD4^+$ counts of less than 200/mm^3 (in particular, less than 100/mm^3), and secondary prophylaxis are essential in AIDS patients with visceral leishmaniasis.[79,81] Monitoring of these patients with PCR proves to be a sensitive and specific method to detect relapses.[82]

As in the case of *Leishmania*, species differentiation of other protozoa by light microscopy is difficult. Clinically important examples include microsporidia and cryptosporidia. The species identification of microsporidia, for instance, is essential because certain species are amenable to antiparasitic treatment, as in the use of albendazole for *Encephalitozoon* spp. Both microsporidiasis and cryptosporidiosis were previously associated with immunocompromised patients, especially in the setting of HIV/AIDS. However, it is now recognized that immunocompetent individuals are not immune to infections by these protozoa.[83] Microsporidial keratitis is increasingly recognized in recent years, sometimes causing outbreaks in otherwise healthy individuals after sports-associated exposure to soil.[84,85] Outbreaks of enteric cryptosporidiosis occurred regularly in different countries of the world, in communities and in institutions, and both the immunocompetent and immunocompromised individuals are affected alike. *Cryptosporidium* has become the commonest protozoa causing waterborne outbreaks.[86] *Cryptosporidium* can usually be detected in faecal specimens easily by acid-fast stains. Light microscopic detection of microsporidia is more problematic because of their small size. Although a number of stains, such as modified trichrome and fluorochrome (using Uvitex 2B or calcofluor white) stains, can be used, their sensitivity and specificity are limited.[87] Given the difficulties in cultivating these organisms *in vitro*, and that immunofluorescent stains do not cover all the common species, molecular techniques remain the method of choice for accurate speciation. Sequencing analysis is often required for species identification of these protozoa especially when pan-microsporidia consensus primers targeting 18 S rDNA are used.[87–91] More thorough characterization of various morphologically similar or identical protozoa by PCR with consensus primers followed by gene sequencing has enabled the discovery of various novel human and zoonotic species such as *Babesia* spp.[92,93]

Ova or adult helminths with similar morphology

Problems of morphological identification are not unique to protozoa. Ova of helminths from different genera or species may have identical morphologies. Sometimes this may be inconsequential when the treatment is identical. Nonetheless, precise speciation is necessary when there are significant differences in clinical consequences, intermediate hosts and control measures, or choices of therapy. An example is the so-called small trematode eggs, which generally refers to trematode eggs of 20–30 μm in length that are commonly found in the Far East.[94] They include ova from *C. sinensis*, *Opisthorchis viverrini*, *Opisthorchis felineus*, *Metagonimus yokogawai*, *Heterophyes heterophyes*, *Haplorchis taichui*, *Haplorchis pumilio*, *Haplorchis yokogawai*, *Metorchis bilis*, *Phaneropsolus bonnei*, *Prosthodendrium molenkampi*, *Metorchis conjunctus*, and *Centrocestus formosanus*. Certain species, such as *C. sinensis* and *O. viverrini*, are carcinogens and accurate identification is essential. Although subtle differences in the egg appearance have been described in the literature, they are often very minor and difficult to discern, especially for microscopists outside endemic areas.[94–99] Given the relatively limited sensitivity of microscopy, one may argue that screening of bile (for which repeated samples may not be readily available in the absence of external drainage) or gallstones with NAAT could be beneficial in endemic areas to detect and treat asymptomatic *Clonorchis* and *Opisthorchis*-infected patients as a preventive measure against cholangiocarcinoma.[100,101]

Another group of trematodes, members of the Family *Fasciolidae*, also contains human pathogens of similar morphologies. Examples include *Fasciola hepatica*, *Fasciola gigantica*, *Fasciolopsis buski*, and the intermediate forms of *Fasciola*. PCR or PCR-RFLP provide definitive diagnosis of these related genera and species.[102–104] With better genetic characterization, the intermediate forms of *Fasciola* is now considered to be a separate species named *Fasciola hepatogigantica*.[105] Similarly, the eggs of *T. solium* and *T. saginata* are indistinguishable. In the absence of the scolices and gravid proglottids, species identification can only be achieved by molecular testing.[106]

Misdiagnosis due to morphological similarities to common parasites

A corollary of the difficulties in accurate speciation by morphology is that parasites may be misidentified as another genus or species. Owing to the similarities between the trophozoites, *Babesia* infection may sometimes be mistaken for *Plasmodium* species.[107] In the past decade, one of the most prominent examples of such misidentification is the recognition of human infections due to *Plasmodium knowlesi*, a simian *Plasmodium* species which normally infects various primates in Southeast Asia. Although *P. knowlesi* has been discovered by Sinton and Mulligan in 1932, human infections have only been rarely described. Natural infection of humans by *P. knowlesi* was first reported in 1965, and sporadic cases occurred in 1971, 1978, and 1998.[108–111] Since 2004, it has increasingly been recognized that *P. knowlesi* is a prevalent form of zoonotic malaria among humans in Southeast Asia, especially in regions of the Malaysian Borneo.[112] Knowlesi malaria accounts for up to 77% of the human cases of malaria in some localities of the Malaysian Borneo.[113] The problem in the accurate diagnosis of knowlesi malaria lies in the fact that the trophozoites resemble other species of human malaria parasites, especially characteristic is the presence of intra-erythrocytic band forms that are typically seen in *Plasmodium malariae*.[114] As a result, about 86–95% of *P. knowlesi* in the blood films were initially identified as *P. malariae*.[112,113] Molecular techniques (primarily using PCR against the small subunit rRNA or other targets) remain the most useful methods for definitive identification of *P. knowlesi*.[115–117] Although most cases of human knowlesi malaria will respond to standard antimalarial treatment, severe disease and fatalities do occur (in contrast to *P. malariae* infection which is typically more benign).[113,118] In addition, the mosquito vectors and natural reservoir hosts of *P. knowlesi* are distinct from other species of *Plasmodium*,[119] the accurate identification of *P. knowlesi* is therefore important both clinically and epidemiologically in assessing the potential for local transmission of the parasite.

Mixed infection not readily discernible by conventional microscopy

Mixed infections due to morphologically similar parasites could be difficult to discern. Under-diagnosis of mixed infection, as in the case of malaria, is therefore a common problem in both residents in endemic countries and returning travellers.[120] This is often attributable to the similar morphology of very young trophozoites and the low-level parasitaemia of one of the co-infecting species. Failure to recognize mixed infection by different *Plasmodium* species may lead to treatment failure.[121–123] The incidence of mixed *Plasmodium* species infection is around 2% based on microscopy.[120] Using molecular diagnostics, a much higher incidence of mixed infection was detected, with over 20% of the malaria cases being co-infections in some endemic areas.[124–129] Therefore, the use of PCR should be considered, at least in the regional reference malaria laboratory setting, to supplement microscopy to confirm the results of speciation and to exclude co-infections. Similarly, mixed infections due to different species of protozoa can only be detected by the use of molecular assays, as in the case of leishmaniasis.[130] The use of multiplex real-time PCR assay is sensitive and specific in detecting different genera or species in one reaction. This technique offers a relatively simple and cost-effective means for the simultaneous diagnosis of multiple parasites that are endemic in a certain area and distinguishes morphologically similar species that can be found in the same clinical specimen (such as blood or faeces).[131–140]

Identification of parasites from histopathological sections

Histopathological identification of parasites requires highly specialized skill and knowledge. This is especially difficult for helminths as many pathologists in the developed countries do not come across such specimens frequently. Identification of helminths to a particular class (Nematoda, Cestoda, or Trematoda) is usually not difficult because each class of parasites has a characteristic basic layout of structures. Identifying them to genus and even species levels is much more challenging.[141] The problem is compounded by the fact that the morphological descriptions of some parasites could be inadequate, and that some fine structures are only discernible by scanning electron microscopy, a facility that is not available in most routine diagnostic laboratories.

Molecular identification of parasites from histological specimens poses special difficulties. To most clinicians and surgeons in the developed countries, parasitic infection is seldom a differential diagnosis in patients with unexplained lesions requiring tissue diagnosis (such as skin and soft tissue masses or space-occupying lesions in the viscera). Unless some foresights are exercised at the time of surgical specimens' collection, most of these specimens will be fixed in formalin or other cross-linking fixatives. Retrospective extraction of nucleic acids from formalin-fixed paraffin-embedded tissues is a major challenge, both in the quantity and quality of nucleic acids, because of cross-linking and fragmentation of nucleic acids in formalin-fixed specimens. Various protocols, including the use of micro-dissection techniques, have been developed to optimize extraction of nucleic acids from these specimens. Commercial kits, some of which are fully automated, are available for extraction from formalin-fixed tissues. Laboratories engaged in molecular studies of histological samples or archival tissues must carefully choose the optimal methods for nucleic acid extraction.[142–148] In recent years, PCR-sequencing has been successfully applied in the definitive identification of helminths in tissue sections, often with the detection of novel or rare zoonotic pathogens which would otherwise be difficult or impossible to speciate from morphology alone, as in the case of human dirofilariasis.[149–152]

Detection of antiparasitic resistance

Unlike bacterial pathogens, the determination of resistance to antiparasitic agents is neither easy nor routinely performed. This is in part attributed to the relative infrequent

occurrence of resistance among most parasites (with the exception of *Plasmodium*), and the difficulties or inability to cultivate most parasites *in vitro*. The ability to detect antimalarial resistance is of great importance, both in the choice of therapeutic agents and in global surveillance of resistance. Traditionally, antimalarial resistance is determined by *in vivo* or *in vitro* studies.[153] *In vivo* studies involved the measurement of parasitological response (level of parasitaemia) to antimalarial treatments, which classifies parasite susceptibility to antimalarials into sensitive and three resistance levels, RI, RII, and RIII. These criteria were initially devised to describe the spectrum of response to standard doses of chloroquine.[154] The interpretation of results could, however, be problematic for drugs with long half-lives.[155] *In vivo* studies also required prolonged monitoring of patients for their responses (14 or 28 days), and could be confounded by the possibility of reinfection in high endemic settings. *In vitro* studies, on the other hand, determined the susceptibility of the *Plasmodium* isolates towards a gradient of concentrations of antimalarial agents, and such techniques require *in vitro* cultivation of *Plasmodium*, which is generally available only in reference laboratories.[153,156]

The identification of target genes of antimalarials and their mutations conferring resistance enabled one to detect resistance by genotypic methods. In addition to being less time-consuming and relatively inexpensive, detection of genotypic markers of resistance also allows large-scale surveillance studies for the study of resistance. Examples of such resistance gene markers include dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) for sulphadoxine-pyrimethamine, *P. falciparum* chloroquine resistance transporter (*pfcrt*) for chloroquine; and *P. falciparum* multi-drug resistance transporter 1 (*pfmdr1*) and multi-drug resistance-associated protein (*pfmrp*) which mediates resistance to multiple antimalarials.[157–159] Recently, single nucleotide polymorphisms in *P. falciparum* chromosomes were identified which may contribute to artemisinin resistance.[160] While such genotypic assays are likely to be out of reach for most routine diagnostic laboratories, they should be considered essential in regional laboratories in endemic countries as part of the surveillance programmes, and in reference centres of non-endemic countries where imported cases of malaria are managed.

Compared to antimalarials, anti-parasitic resistance in other protozoa, and especially the helminths and ectoparasites, generally received much less attention. The significance of detecting anti-parasitic resistance cannot be overstated. For many parasitic infections, especially the helminthiases, the number of effective agents or classes of agents for each group of parasites is relatively limited. Development of resistance to the first-line agents means not only treatment failure for individual patients, but also compromises various mass treatment programmes in endemic areas. Although certain genetic and metabolic mechanisms have been implicated in drug resistance in protozoa and helminths (such as nitroimidazole resistance in *Giardia* and *Trichomonas*, benzimidazole resistance among soil-transmitted helminths, and potential praziquantel resistance in *Schistosoma*), many of these have not be utilized as part of the routine diagnostics.[161–164] A problem clinically much more relevant and disturbing lies among the ectoparasites, where resistance to some first line insecticides has been prevalent. One of the best studied example is the resistance of *Pediculus humanus capitis* (head louse) to DDT, lindane, pyrethroids, and malathion, which is now prevalent in many countries. Additionally, multiple or cross resistance to different agents is not rare, making clinical management of these infestations more problematic.[165–169] As in the case of head louse, pyrethroid resistance has also been described in *Sarcoptes scabiei*, the scabies mite.[170] In addition to pyrethroids, resistance to lindane and even ivermectin has been described in *S. scabiei*.[171–173] Molecular assays have been developed to detect mutations in the *kdr* (knockdown resistance) and *Vssc* (voltage-sensitive sodium channel) genes which account for pyrethroid resistance in lice and scabies mites, which could be useful for surveillance purposes.[174–176]

Screening of at-risk individuals

As in the case of transfusion-transmitted infections, the use of molecular assays may potentially be beneficial in the prevention and management of transplant-related parasitic infections (Table 2). Such assays may serve three roles in such infections. Firstly, although NAAT screening of donor tissues is theoretically possible, this approach has not been vigorously tested. Potential drawbacks of this approach include sampling errors resulting in false negative results, and the significantly longer turnaround time as compared to serological testing, the latter being especially important in cadaveric organ transplantations. As such, serology remains the mainstay of screening for most infections (such as toxoplasmosis and Chagas disease).[177,178] Secondly, NAAT can be used for monitoring and diagnosing reactivations of parasitic infections using either blood or tissue biopsy samples.[177–181] This may circumvent some of the problems associated with serological tests, such as inadequate serological responses while on immunosuppression, and differentiating active from past infections in endemic areas. Thirdly, asymptomatic parasitic infections are common for a number of protozoal and helminthic infections, such as visceral leishmaniasis and strongyloidiasis.[182–184] In addition to the possibility of donor-transmitted parasitic infections, reactivation of subclinical infection is also possible. Although pre-transplant screening of recipients with serological tests is the usual practice,[185] further studies should explore the role of NAAT in predicting the need for prophylaxis of subclinically infected transplant recipients.

The potential role of NAAT in early diagnosis and donor/recipient screening is not limited to organ or haemopoeitic stem cell transplantations. Patients requiring intensive immunosuppressive therapy, especially the use of corticosteroids, are at risk of reactivation of strongyloidiais.[186] A more comprehensive programme for screening asymptomatic infections by these parasites should be considered in centres caring for immunocompromised patients, according to the local prevalence of these parasitic infections.

Table 2 Transplant-related parasitic infections and potential roles of molecular methods

	Donor-derived infection	Post-transplant reactivation	Type of transplantation reported	Conventional laboratory screening method (donor)	Actions if donor screen positive	Conventional laboratory screening method (recipient)	Potential role and limitations of screening using molecular methods	Potential role of molecular methods in diagnosis of post-transplant disease	Reference
Babesiosis	Not reported	Not reported	Kidney, heart, HSCT (few case reports of *de novo* or blood transfusion-related infection in transplanted patients only).	Microscopy and/or serology in at risk donors in some centres.	Avoid use of blood products.	Microscopy and/or serology in at risk recipients in some centres.	PCR has greater sensitivity compared with microscopy and or serology allowing detection of low-level parasitaemia. However, a complete guarantee of safe blood product is not possible even with PCR.	Detection of low-level parasitaemia and avoid misdiagnosis of *Plasmodium* infection which can occur with microscopy. However, very low level of parasitaemia may still be missed.	32,39,200–205
Malaria	Yes	Not reported	HSCT, liver, kidney, heart transplantations.	Microscopy and/or serology in at risk donors in some centres.	Avoid transplantation during active infection; use of preventive antimalarial reported in a case report.	Microscopy and/or serology with at risk recipients in some centres.	Detection of low level infection (50 parasite/mL), but the PCR must be designed to cover all *Plasmodium* species. However, complete guarantee of safe blood products is not possible even with PCR.	PCR has higher sensitivity in the detection of dual infections and low level infections. Post-exposure monitoring may allow early detection of low-level parasitaemia.	206–212
Chagas disease	Yes	Yes	Highest risk of donor-derived infection and reactivation in heart transplant recipients. Other reports include kidney, kidney-pancreas, bilateral lung transplantation, and HSCT.	Serology; at least two out of three serological tests of different methodology.	Avoid high risk organs (especially heart). Close monitoring in post-transplant in recipients of other tissue type.	Strout's method and/or culture and/or biopsy and/or serology.	Detection of latent/occult infection in donor leading to avoidance of transplantation of high risk tissue. Chronic disease associated with a very low level of parasitaemia may not be detected by PCR and serology may be more helpful in these groups. Identification of infected donors enables close monitoring of recipient during post-transplant period.	PCR is more sensitive than haemoculture or serology. Positive PCR often precedes development of symptomatic infections, thus it is useful for monitoring potential donor-derived infections or reactivations. Pre-emptive treatment can be started during early phase of infection when PCR becomes positive. However, PCR may remain positive with nonviable parasite which may confuse the clinical picture.	179, 213–218
Visceral leishmaniasis	Yes	Yes	Over 110 cases described, predominantly in kidney transplant	No recommendations on donor screening.	Avoidance of using HSCT in these patients. No clear	Serology in at risk recipients in some centres.	Detection of low level occult infection may be possible by PCR which has higher sensitivity.	PCR of bone marrow or peripheral blood has a quoted sensitivity of 70–90%. PCR combined	219–225

(continued)

Table 2 Continued

	Donor-derived infection	Post-transplant reactivation	Type of transplantation reported	Conventional laboratory screening method (donor)	Actions if donor screen positive	Conventional laboratory screening method (recipient)	Potential role and limitations of screening using molecular methods	Potential role of molecular methods in diagnosis of post-transplant disease	Reference
			recipients. Other reports in liver, heart, lungs, kidney, pancreas transplantation, and HSCT recipients.		recommendations for SOT.		However, reactivation in seropositive recipients with latent infection is possible and may not have positive PCR on screening.	with microscopy and/or biopsy and/or culture have been suggested for early diagnosis of asymptomatic leishmaniasis infection during post-transplant period.	
Toxoplasmosis	Yes	Yes	Donor-derived infections mostly associated with heart transplantations. Liver, kidney, and intestinal transplant-derived infections have been reported. Reactivations of toxoplasmosis have been described in HSCT patients.	Serology	Chemoprophylaxis is recommended in high risk recipients (e.g. heart transplantation from an infected donor; Donor-negative-recipient-positive serology HSCT recipients).	Serology	PCR may be useful in assessing haemopoietic stem cells obtained from donors where serology results suggest potential recently acquired infection.	PCR testing of blood, CSF or BAL in combination with clinical, radiological, and/or histological evidence. High-risk recipients who has delayed prophylaxis is suggested to have weekly monitoring using PCR.	205,226–233
Strongyloidiasis	Yes	Yes	Reactivations are more common and have been reported in heart, kidney, liver, lung transplantation, and HSCT. Few donor-derived infections in intestine, pancreas, and kidney transplantation reported.	No recommendations on donor screening.	Pre-transplantation treatment in infected donor for intestine, pancreas, and kidney may reduce risk of donor-derived infection. Potential pre-emptive treatment in recipient.	Serology and/or 3 stool specimens for microscopy.	PCR has greater sensitivity and specificity. Comparatively, serology has lower sensitivity and is prone to cross-reactivity in patients with other helminthic infections.	PCR detects the presence of low level of *Strongyloides* not detectable by microscopy or serology, enabling early treatment and prevention of hyper-infection syndrome.	234–247

HSCT, haemopoietic stem cell transplantation; PCR, polymerase chain reaction; SOT, solid organ transplant; CSF, cerebrospinal fluid; BAL, bronchoalveolar lavage.

Conclusion: Molecular diagnosis or alternatives?

Although molecular diagnosis has always been touted as a means for rapid diagnosis in many fields of diagnostic microbiology, this may not necessarily apply to parasitology. The turnaround time for microbial culture is often long in mycobacteriology, mycology, and virology, hence molecular assays are excellent solutions for rapid diagnosis. In contrast, diagnostic parasitology, with few exceptions, does not rely on *in vitro* cultures. With the need for transportation to centres with NAAT facilities, specimen preparation, nucleic acid extraction and amplification, conventional staining and light microscopy generally offers a similar, if not faster, turnaround time. This is especially important in off-hour settings where personnel for molecular diagnosis may not be readily available. Another consideration is that routine microscopy is a catch-all technique whereby the presence of any parasite in the clinical specimen can be detected. On the contrary, NAAT methods are targeted only towards the species covered by the primers, though the use of multiplex PCR does increase the spectrum of detection. For these reasons, molecular methods cannot as yet replace microscopy as routine diagnostic tests, but could be extremely useful when a specific parasite or groups of parasites are to be studied.

The specific identification of parasites by NAAT relies on the availability of accurate gene sequence information, and this in turn depends on the precise morphological identification of parasites. In contrast to most bacterial and viral agents, the genetic information on many parasites remains limited. The utility of molecular method for identification of parasites is sometimes limited by the lack of such information. Similarly, when gene sequences are used to identify new species of parasites, their morphology should always be considered simultaneously, lest wrong designation to new genera or species may occur.[187]

Despite the many benefits of molecular assays, their adoption by smaller local laboratories, even in developed countries, requires careful considerations. If the number of specimens received for testing is low, it would be more cost-effective for these specimens to be tested in regional or reference centres. The choice of the tests to be performed depends largely on the spectrum of parasitic diseases encountered in a particular centre. Although serological tests have been described for many parasitic diseases, many of these assays were developed in-house and therefore they are not readily available to most laboratories. The availability of molecular assays allows non-research centres to possess the ability to diagnose less frequently encountered parasites, provided that the tests are well validated. The validation of newer NAAT assays poses problems which are not unique to parasitology. The determination of the performance characteristics (sensitivity, specificity, positive and negative predictive values, and so on) requires comparison of the new assays with a diagnostic gold standard. As noted at the beginning, microscopy has been the gold standard in most areas of diagnostic parasitology. NAAT assays are generally considered to be more sensitive than conventional microscopy. The use of microscopy as a gold standard may therefore underestimate the performance of NAAT. This issue is also encountered in other areas of diagnostic clinical microbiology when NAAT was first introduced, an example being the use of NAAT for the diagnosis of *Chlamydia trachomatis* and herpes simplex virus infections when compared to bacterial and viral culture. An ''expanded gold standard'' approach has been adopted which employed more than one diagnostic test and the clinical presentations as the reference standard.[188–191] While this approach has worked reasonably well for many bacterial and viral infections, its application for parasitic diseases is faced with more difficulties. Firstly, multiple diagnostic approaches have to be adopted which is not always included in all studies. These include clinical manifestations, conventional microscopy, serology (antigen and/or antibody detection), and even tissue biopsies. For microscopy, multiple specimens may have to be collected serially to overcome the inherent limitations of the test. Exclusion of alternative diagnoses by appropriate laboratory testing should also be performed. Secondly, as in the case of most new NAAT assays, many of the adjunctive serological assays are also developed in-house, and their suitability as part of the expanded gold standard is uncertain. Understandably, not all new NAAT assays published have undergone the necessary stringent validation processes. Ongoing evaluation of the new assays in different geographical areas and different patient populations is essential, as well as to establish the clinical significance of positive results.

Author contributions: SSYW reviewed the whole literature relevant to the paper and prepared the manuscript. K-YY initiated and set the focus of this review. SCYW and K-YY critically reviewed and revised the manuscript. RWSP carried out the study on the local molecular epidemiology of *Clonorchis sinensis*, designed new primers and summarized the literature in Table 2. KSCF and SC collaborated in the local study on the molecular epidemiology of *Clonorchis sinensis* and reviewed the manuscript.

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