

Chapter 2

Molecular Diagnostics of Parasitic Infections

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Abstract DNA-based methods have been available for many years in most clinical microbiology laboratories. However, until recently, these tools were not routinely exploited for the diagnosis of parasitic infections. Laboratories were reluctant to implement PCR, not knowing how to incorporate such an approach in the algorithm of tools available for the most accurate diagnosis of a large variety of parasites. This was especially noticeable in the diagnosis of intestinal parasitic infections, where the diversity of parasites that one can expect in most settings, is much smaller than the parasitological textbooks suggest. Therefore, the classical algorithm, which is based on population, patient groups, use of immuno-suppressive drugs, travel history, etc., is also applicable to the decision on whether or not to perform additional techniques when a multiplex PCR panel is used as a first line diagnostic.

Keywords Protozoa · Helminths · Malaria · *Giardia lamblia* · Intestinal parasites

2.1 Introduction

Traditionally, laboratory diagnosis of parasitic infections is based on the examination of microscopical slides and, as a consequence, highly depended on the expertise and skills of the microscopist. Although various staining and concentration techniques have been introduced to enhance the specificity and sensitivity, the diagnostic performance is known to be low and time-consuming. Very soon after the first publication on in vitro DNA amplification in a polymerase chain reaction (PCR), a breakthrough was predicted in molecular parasitology and in the diagnosis of parasitic infections. Until that time, specific DNA probes were used in fundamental research, but diagnostic applications had been hampered by the limited sensitivity of these hybridization techniques. In 1995, a number of publications for

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DNA-based methods for the detection and identification for a range of parasitic infections were reviewed by Judith Weiss. At that time, the use of PCR was still limited and most of the research was done on malaria, *Leishmania*, trypanosomes and *Toxoplasma*, which are all tissue parasites. Moreover, except for toxoplasmosis, experience with PCR performed on DNA that had been isolated directly from patient material was scarce.

In the last 10 years, many diagnostic laboratories have been provided with the facilities to perform molecular diagnostics. The introduction of real-time PCR reduced the problems related to the contamination of samples with PCR products and it became relatively easy to combine multiple DNA targets in one multiplex assay. In addition, the implementation of automated DNA isolation methods made it possible to use DNA-based detection techniques in a high-throughput format in diagnostic routine laboratories.

Molecular detection, differentiation and genotyping methods have been described for a large number of parasitic infections. These methods are now performed in both diagnostic and research settings. This chapter is a selection of the most clinically relevant parasitic infections in industrialized countries, with an emphasis on the use of molecular diagnostics in a routine setting.

2.2 DNA Isolation

Without a proper method for DNA isolation, DNA amplification techniques cannot be used. Especially when parasitic DNA has to be extracted from faeces, proper DNA extraction is extremely important. When setting up a PCR, it is essential to ensure that the target DNA will be released from the parasitic stage (e.g., cysts, eggs or spores) with the isolation method used and from the relevant clinical material. Moreover, amplification of DNA isolated from faeces is notoriously difficult due to the presence of factors that can inhibit the amplification reaction (see Vol. 1, Chaps. 5 and 6). Heating the faecal sample and the addition of absorbent substances such as polyvinyl polypyrrolidone (PVPP) during the DNA isolation procedure or the addition of inhibition factor-binding substances, such as bovine serum albumin (BSA), in the PCR may be used to prevent inhibition of the amplification. However, it always remains important to use an internal inhibition control in each reaction. For example, Phocine Herpes Virus (PhHV) can be added during the DNA isolation procedure, after which a specific PhHV PCR, in multiplex with the PCR(s) for the target(s), can be performed. Nowadays, more and more DNA isolation kits and protocols for automated DNA isolation systems are commercially available. As highlighted above, with the introduction of a new DNA isolation protocol or when changing an existing protocol, it is vital to consider whether the DNA of the intended target(s) is released. This can be done by testing known positive samples. Both in our own experience and as published by others, preservation of faeces with

formalin or saline acid formalin (SAF) has an enormously negative effect on the specific amplification of DNA. This effect is enhanced by the time that the sample has been stored in the fixative.

2.3 Intestinal Parasites

2.3.1 Protozoa

Entamoeba histolytica is the cause of amoebic colitis, amoebic dysentery and amoebic liver abscess and is responsible for an estimated 100,000 deaths per year. Until about 25 years ago, differences in clinical symptoms were explained by the existence of pathogenic and non-pathogenic strains of *E. histolytica*. Biochemical, immunological and genetic differences between these “strains”, however, are so large that it is now generally accepted that they are in fact two different species, named *E. histolytica* and *Entamoeba dispar*. Because the potentially invasive *E. histolytica* is morphologically indistinguishable from the non-invasive *E. dispar*, microscopic examination of faecal samples only, is not suitable for a definitive diagnosis of *E. histolytica* infection and additional methods, such as the detection of specific antigens or specific DNA sequences are necessary. Although antigen detection is specific, its sensitivity is too low, when compared with PCR, for use in patient care.

Giardia Lamblia infections are very common, occur globally and are seen as one of the most important non-viral causes of diarrhoea in industrialized countries. Since the time of Anthony van Leeuwenhoek, the diagnosis has been based on microscopic investigation of (multiple) faecal samples using concentration techniques. The detection of *Giardia* antigens by ELISA has been accepted in recent years as a cost-saving alternative. PCR targeting the SSU rDNA has excellent sensitivity and specificity when compared to microscopic and antigen based detection strategies. *Giardia* can be divided into various genotypes (assemblages) on the basis of Restriction Fragment Length Polymorphism (RFLP) analysis and DNA sequence analysis of several (household) genes. However, the clinical significance of the various assemblages is still unclear and therefore, these genotyping methods are used in epidemiological and basic research settings only.

Cryptosporidium-associated diarrhoea is best known for the severe symptoms in AIDS patients during the HIV/AIDS epidemic in the 1980s and 1990s. Subsequently, improved diagnostic methods have been developed. Probably due to this awareness, *Cryptosporidium* is now also known as the cause of large water- and food-borne outbreaks of gastroenteritis in immunocompetent individuals. Modified acid-fast staining techniques are often used for the detection of *Cryptosporidium* oocysts in stool. However, the sensitivity and specificity appear to be low and proper identification is very dependent on the experience and skills of the microscopist. Although immunological detection methods are available, these are not as

well accepted as in the diagnosis of *Giardia* infections due to low sensitivity and specificity. PCR based techniques have been proven to be a sensitive and specific alternative for the detection of *Cryptosporidium* in faecal samples. In a study including 950 patients from the Groningen region in the Netherlands, who visited their family doctor with gastrointestinal symptoms, *Cryptosporidium* was demonstrated using real-time PCR in 20% of children under five.

PCR based techniques have also been shown to have a high sensitivity for the detection of other coccidia-like infections, such as *Cystoisospora belli* (*Isospora belli*) and *Cyclospora cayetenensis*. Another example in which PCR is a worthwhile alternative in the diagnosis of opportunistic pathogens is microsporidiosis. Microscopic detection of the very small spores of different microsporidium species in faecal samples, even after additional staining techniques, has proven to be extremely difficult and time-consuming. Amplification of microsporidium-specific DNA has been shown to be a sensitive and specific tool in the diagnosis of microsporidium infections. Probably due to the increased attention on microsporidiosis during the AIDS epidemic and the improvement of diagnostic techniques, such infections are now being diagnosed more frequently in transplant patients, children, the elderly and in travellers.

Dientamoeba fragilis was first described in 1918 as a commensal living amoeba of the gastro-intestinal tract of humans. Subsequently, through antigen based and ultrastructure studies and through the analysis of the 16S ribosomal RNA, the organism was classified as a flagellate, though a flagellum is missing. Since its discovery, the pathogenicity of this organism has remained controversial. In recent years, several authors have published on the clinical significance of *D. fragilis* as a cause of gastrointestinal distress, unfortunately, a consensus on the pathogenicity is lacking. One of the issues preventing progress is that many *D. fragilis* infections remain without symptoms. Until recently, a cyst stage of *D. fragilis* was not recognized. Therefore, the fragile trophozoites were detected and identified by microscopic examination in fresh stool or by the use of fixatives and permanent staining techniques. The sensitivity of a single microscopic examination is not high, because the day-to-day variation of *D. fragilis* trophozoites in the faeces seems even more irregular than observed in other intestinal protozoan infections, such as *G. lamblia* and *E. histolytica*. Immunological techniques for the detection of *D. fragilis* are not available. Real-time PCR has been proven to be a highly sensitive and specific alternative to the diagnosis of *D. fragilis*, in which the influence of the day-to-day variation of the results is greatly reduced. *Dientamoeba* DNA can be detected successfully with qPCR, even after storage of the faecal samples for up to eight weeks at 4 °C, without loss of sensitivity. Genetic variations of the small subunit ribosomal RNA (SSU rRNA) gene and of the internal transcribed spacer 1 (ITS1) of *D. fragilis* have not been associated with the occurrence of clinical symptoms. Furthermore, in a recent multicentre case-control study in the Netherlands, *D. fragilis* was detected more often in controls as compared to diarrhoeal cases, which has led many laboratories to remove *D. fragilis* from their primary screening panel.

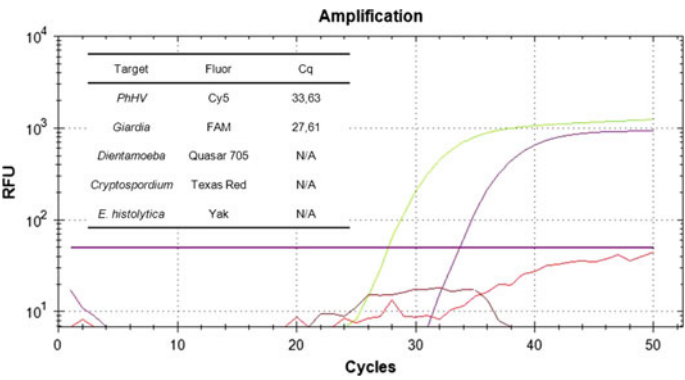


Fig. 2.1 Example of amplification curves of *Giardia* and the internal inhibition control in the same sample in a multiplex real-time PCR for the detection of *E. histolytica*, *D. fragilis*, *G. lamblia*, *Cryptosporidium* and PhHV DNA (N/A = Not detectable)

Blastocystis is the most common intestinal parasite in the faeces of both patients with gastrointestinal complaints and in asymptomatic individuals. It is thus, even more so than with *D. fragilis*, unclear whether it is necessary to detect this parasite. PCR-based techniques display a higher sensitivity when compared to microscopy and culture and provide the possibility of performing sub-typing by means of DNA sequence analysis. Factors that seem to be associated with the clinical significance of *Blastocystis* are the parasite load, the genotype or subtype, and the use of antimicrobial treatment (Fig. 2.1).

2.3.2 Helminths (Worms)

Strongyloides stercoralis is endemic in many tropical and subtropical regions. It is estimated that worldwide 50–200 million people are infected, with prevalence rates as high as 50%. *S. stercoralis* behaves differently from other intestinal nematode infections. Rhabditiform larvae are excreted in the faeces, but may already develop to infectious filarial larvae in the intestines. These larvae can then penetrate through the intestinal mucosa or the perianal skin, which results in a long-term cycle of auto-infection that can continue for many decades. Chronic strongyloidiasis may result in hyper infection, which is often seen in immunocompromised hosts, such as transplant patients, patients receiving chemotherapy and patients treated with corticosteroids.

The laboratory diagnosis of *S. stercoralis* is mainly based on serology and on the detection of *S. stercoralis* larvae by microscopic examination of faecal samples. Microscopy is very labour intensive, particularly in chronic infections. The number of larvae is very small and even after formalin-ether concentration (the Baermann method) or coproculture, sensitivity is low and multiple samples have to be

examined in order to achieve the appropriate level of sensitivity. A *S. stercoralis*-specific qPCR was described, with very high specificity, using a large panel of DNA controls and faecal samples. The primer and probe set is based on the 18S ribosomal RNA gene sequence and turned out to be 10 to 100 times more sensitive in comparison with primers and probes designed on the cytochrome c oxidase subunit I gene and a *S. stercoralis*-specific sequence.

For various other soil-transmitted helminth (STH) infections, such as *Ascaris* and hookworm (*Necator americanus* and *Ancylostoma duodenale*), PCRs targeting of the Internal Transcribed Spacer 2 (ITS2) sequence have been developed, but these are still mainly used for epidemiological studies in endemic areas. The occurrence of these infections is very limited in the Netherlands (see also “Algorithm faecal examination”) and infections are often very mild. In addition, these worms have a relatively short life span. It is important to realize that several of the isolation methods that have been published were proven to be unable to release DNA from *Trichuris trichiura* eggs. As a consequence, until recently, PCR for the detection of this parasite could not be integrated into a multiplex panel (Fig. 2.2).

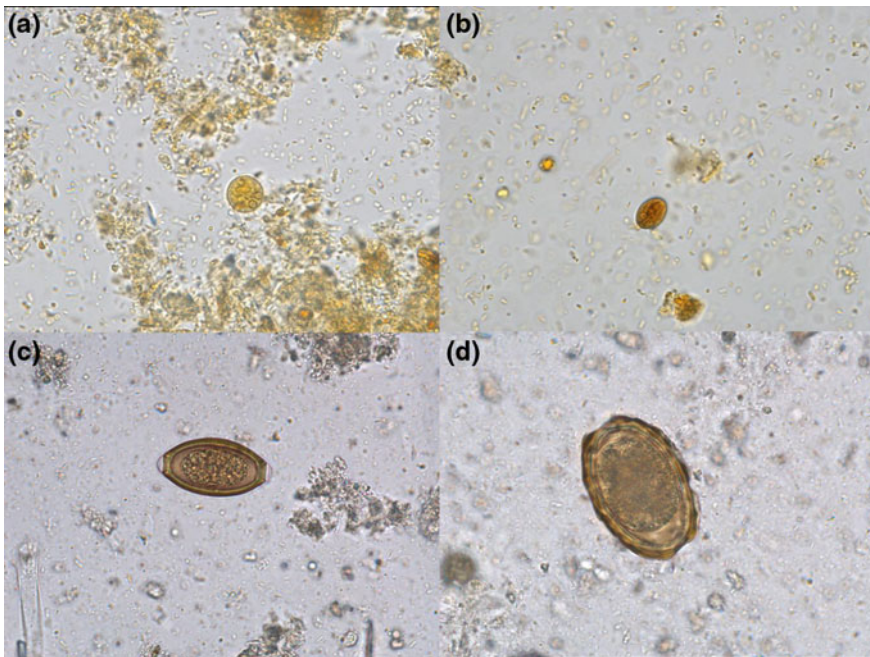


Fig. 2.2 Example of protozoan cysts: *Entamoeba histolytica*/*Entamoeba dispar* (a), *Giardia lamblia* (b) and worm eggs *Trichuris trichiura* (c), *Ascaris* (d). Photos are courtesy of Eric A.T. Brienens, Department of Parasitology, Leiden University Medical Center

2.3.3 Algorithm for Faecal Examination

Conventional methods for the detection of intestinal parasites have their limitations in specificity and sensitivity. This means that species-specific techniques or modifications for each individual organism are necessary to attain the most optimal diagnosis. Multiplex real-time PCR, however, provides a sensitive and specific detection for each of these parasites.

Which PCRs are used in the diagnosis of intestinal parasitic infections is very dependent on the patient population of which the laboratory receives faecal samples. However, the choices that are made do not differ substantially from those used in the decision on the use of additional concentration and staining methods to improve the sensitivity of the detection of specific parasites using microscopy. After all, a coproculture or Baermann for the concentration of *Strongyloides* larvae, for example, is used only when the travel or clinical history provides indications to do so. In the same way, one or more multiplex PCRs can be chosen. These can be supplemented with either PCR(s) for additional targets and/or additional species-specific microscopic methods.

Examples of various target combinations in specific groups of patients are shown in Table 2.1.

Table 2.1 Examples of targets in different patient populations

Adults <i>Entamoeba histolytica</i> ^d <i>Giardia lamblia</i> <i>Cryptosporidium</i>	Children <i>Dientamoeba fragilis</i> ^e <i>Giardia lamblia</i> <i>Cryptosporidium</i>
Travellers <i>Entamoeba histolytica</i> ^d <i>Giardia lamblia</i> <i>Cryptosporidium</i> <i>Strongyloides stercoralis</i> <i>Schistosoma</i> ^b <i>Cyclospora cayetanensis</i> ^b <i>Cystoisospora belli</i> ^b <i>E. bienersi</i> ^c	Immunocompromised patients <i>Strongyloides stercoralis</i> ^a <i>Cryptosporidium</i> <i>Cystoisospora belli</i> <i>Enterocytozoon bienersi</i> <i>Encephalitozoon</i> spp.

^aRecommended before treatment with corticosteroids is started
^bIn travellers to high-risk areas
^cImportance in travellers has been suggested
^dRecommended before diagnoses of M. Crohn is made
^eClinical significance unclear

2.4 Blood and Tissue Parasites

2.4.1 Protozoa

Malaria

Although the number of malaria cases in non-endemic areas is decreasing, the diagnosis of malaria remains a challenge for diagnostic laboratories. The potential fatality of malaria tropica in a patient with fever returning from the tropics makes it necessary to put all other work aside to exclude or confirm an infection with *Plasmodium*. In most laboratories, microscopy and antigen detection are performed as the standard diagnostics, however, PCR has been accepted as a highly sensitive and specific method, in particular in the detection of sub-microscopic infections. Nowadays, PCR can be considered as the gold standard for the differentiation of *Plasmodium* species and for the detection of mixed infections. Until now, malaria PCR is not often performed as a first line diagnostics tool, it is merely being performed in a number of reference centres, for the confirmation of microscopic findings. This limited application is mainly the result of logistics; in most laboratories the microbiological and haematological analytical staff run 24-hour shifts and those specialized in molecular diagnostics usually do not. The 18S rRNA gene is used as the target in conventional and, more recently, real-time PCRs for the detection and differentiation of the four human malaria parasites *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. Recently, human infections with the “monkey malaria” parasite *P. knowlesi* have been described and real-time PCRs, targeting the 18S rRNA gene of this parasite, have been published. Because resistance to the various anti-malarial drugs is an ever increasing problem, various techniques for the detection of mutations in several genes and the number of copies of genes involved in the metabolism of the various anti-malarial drugs in the parasite are applied in the research setting and in epidemiological studies. Examples of such genes are the chloroquine resistance Plasmodium transporter gene (Pfcr) and the Multi-Drug Resistant Plasmodium falciparum gene 1 (Pfmdr1).

Leishmania

The diagnosis of cutaneous and visceral leishmaniasis import infections has traditionally been based on the extensive and intensive microscopic examination of biopsy material or bone marrow by experienced laboratory staff. This is very labour intensive and can be extremely difficult. Nucleic acid amplification methods, such as (real-time) PCR, offer a very sensitive and specific diagnostic alternative. Moreover, these methods give the possibility of species differentiation which is important for the choice of treatment. A genus-specific qPCR based on the SSU rRNA gene is used for the detection of *Leishmania*-specific DNA. The higher sensitivity of molecular diagnostics makes lengthy microscopic examination unnecessary. In cases of suspected visceral leishmaniasis, PCR on blood instead of

more invasive bone marrow examination is a worthwhile alternative, in addition to serological testing. Research in Sudan showed that with conventional PCR, *Leishmania* DNA is detectable in blood in 70% of the proven leishmaniasis patients. This sensitivity is even greater when qPCR is used in which only a very small target is amplified.

The taxonomy of *Leishmania* species is complex and the nomenclature of the different types of complexes is subject to change. This makes it sometimes difficult to compare differentiation techniques. Depending on the travel history, various PCR-RFLP and DNA-sequencing methods based on different targets, such as Internal transcribed spacer sequence (ITS), Heat Shock Protein 70 (HSP70), T2B4 repeat, 7SL and mini-exon DNA, are used for the differentiation of the different species and species complexes. DNA-sequencing of larger targets with higher

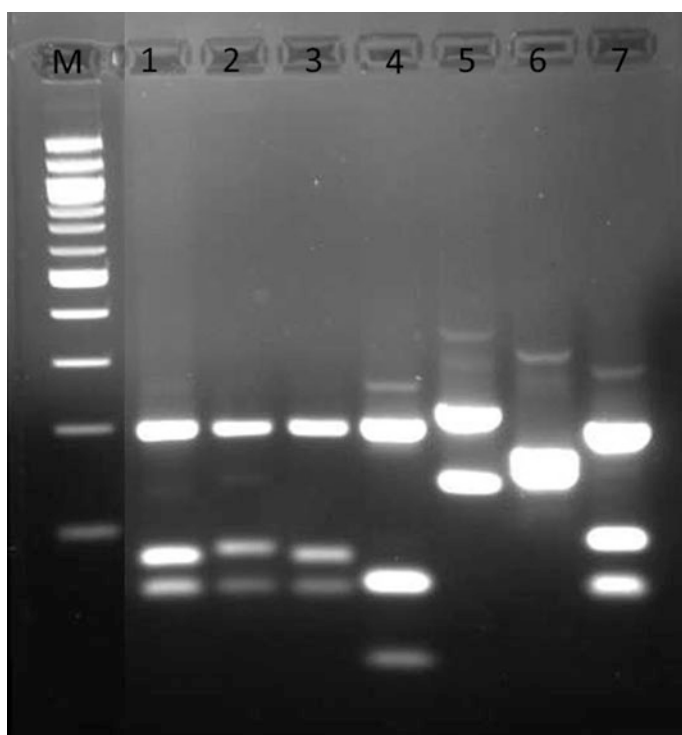


Fig. 2.3 Restriction Fragment Length Polymorphism (RFLP) analysis. Fragments Pattern of a *Leishmania* Internal Transcribed Spacer PCR product after digestion with Hae III. 100 bp marker (M), no difference between *L. infantum* and *L. donovanni* (Lane 1–3), unique patterns for *L. tropica* (Lane 4), *L. major* (Lane 5). No difference within species belonging to the *L. Viannia* subgenus (Lane 6), *L. mexicana* (Lane 7)

genetic diversity, such as HSP70, results in higher resolution of the different species as compared to PCR-RFLP of smaller targets, e.g. ITS (Fig. 2.3).

Toxoplasma

The laboratory diagnosis of toxoplasmosis is primarily based on serology. The detection of specific IgG and IgM antibodies in otherwise healthy people will usually be sufficient for a toxoplasmosis diagnosis or for the determination of the immune status. When immunocompromised, in both ocular toxoplasmosis and congenital toxoplasmosis, this is often much more complicated and molecular diagnostics may complement the serological data. For example, in countries where serological screening for toxoplasmosis in pregnancy is common, *Toxoplasma* PCR is applied on amniotic fluid in cases of seroconversion. Real-time PCR on plasma also shows a simple, rapid and highly sensitive detection method for the early diagnosis of disseminated toxoplasmosis in bone marrow and organ transplant patients. In literature, a large number of DNA targets are described in *Toxoplasma*-specific PCRs, such as the B1, P30 and 18S ribosomal DNA gene and, more recently, a 529 bp-*Toxoplasma*-specific repeat. A comparison of the sensitivity and specificity of the different targets is difficult, because they are highly depended on the size of the PCR product, the primers, the detection probes and on the amplification and detection methods used.

2.4.2 Helminths

Schistosoma

The adult *Schistosoma* worms reside, depending on the species, in the blood vessels, around the intestines or around urinary bladder. Therefore, these are blood-borne parasites, strictly speaking. However, the eggs are excreted through the intestinal wall into the faeces by *Schistosoma mansoni* or through the bladder wall into the urine by *Schistosoma haematobium*. The number of eggs, especially for travellers, is mostly very low. Therefore, larger amounts of faeces and/or urine are concentrated to increase the sensitivity of the microscopic examination. Because of the mostly mild infections in the non-endemic setting, serological testing is more sensitive. In endemic areas, *Schistosoma*-specific PCR, targeting the internal transcribed 1 (ITS-1) sequence on DNA isolated from a small amount of urine or faeces, proved more sensitive than microscopic examination. Recently, a highly sensitive method of detection for *Schistosoma* DNA, with a PCR targeting a 121 bp tandem repeat sequence, has been reported in plasma as well.

2.5 Concluding Remarks

Multiplex real-time PCR provides an extremely sensitive and specific diagnostic alternative for labour-intensive microscopy in clinical laboratory practice. The number of laboratories that implement real-time PCR for the detection of parasitic infections is increasing. This applies in particular to diarrhoea-causing protozoa, which is often combined with a bacterial and a viral enteritis panel, into a total panel. In the western setting, the pathogenic parasites *E. histolytica*, *G. lamblia* and *Cryptosporidium* make up the vast majority of infections that are detected by microscopy. In travellers, additional parasites are found by microscopy in a small minority of cases, especially in those who have travelled to areas with high risk. Additional diagnostic methods for the detection of parasitic infections that are not included in the “standard” faecal PCR panel can thus be restricted to a selected group of patients. The implementation of molecular diagnostics obviously also calls for quality assurance. These initiatives have been taken and regular assessment schemes are available. Molecular diagnostics of less common parasites and more extensive genetic characterizations are carried out in various research centres. All over the world, including in developing countries, real-time PCR is available in a growing number of research centres and molecular diagnostics of parasitic infections is applied in large-scale epidemiological and fundamental research.

In the future, variations on microarrays and next generation sequencing platforms will undoubtedly become more user-friendly and more applicable in a diagnostic setting. This will enhance the use of DNA-based diagnostics in the microbiological diagnostic laboratory.

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