Molecular detection of intestinal parasites for clinical diagnosis and epidemiology

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CHAPTER 1

GENERAL INTRODUCTION

INTRODUCTION

Since the late nineteenth century a vast amount of scientific data has been compiled on the causal relationship between parasites and diseases in man. It was anticipated that as a consequence of the overall medical progress and global initiatives in eradication programs, infectious parasitic diseases would eventually become something of the past. Malaria, sleeping sickness, visceral leishmaniasis, Chagas' disease, river blindness and guinea worms are some examples of diseases on which international cooperative intervention programs have focused in the 20th century (Hopkins et al., 2005, 2008; Fèvre et al., 2006; Yamagata & Nakagawa, 2006; Alvar et al., 2006). In developed countries, many parasitic diseases have been eradicated or their prevalence has declined significantly. However, despite enormous efforts in eradication programs, it is striking to observe that 'classical' parasitic diseases continue to re-emerge in other parts of the world (Molyneux, 2004; Hotez et al., 2007; Stratton et al., 2008). Not only do diseases re-emerge, other parasitic infections (e.g. microsporidia and *Cryptosporidium*) have been described for the first time only recently (Lashley & Durham, 2007; Topazian & Bia, 1994). Factors that have contributed to the decline or the emergence of human pathogenic parasites include changes in sociocultural patterns and human behaviour as well as the overall expansion of human activities and their impact on ecosystems. Nevertheless, the exact reasons are often complex and interrelated (Cohen & Larson, 1996; Gubler, 1998; Lashley, 2003).

The human intestinal tract may be a habitat to a variety of parasites, ranging from microscopic small microsporidia to meters long tapeworms. Observations on the most common and larger intestinal worms were already described in the earliest civilizations (Cox, 2002). Smaller intestinal parasites that were not visible by eye could only be observed after the invention of the microscope. In the seventeenth century Antonie van Leeuwenhoeck was the first to observe these little animals (“dierkens”), which were later described as *Giardia lamblia*. The invention of the microscope caused a major breakthrough in parasitology and has ever since been the classical tool for identifying parasites. Only a small portion of all intestinal parasite species may cause serious health problem, but most live in the gastro-intestinal tract without causing significant harm to its host. In some way they may even have a positive effect on the host's immunological system (Yazdanbakhsh et al., 2002).

Despite that our understanding of parasites has improved enormously over the last decades, the tools that are used for parasite detection have remained largely the same. Laboratory diagnosis of intestinal parasite infections still depends mainly on microscopical examination of stool samples for the identification of helminth eggs and protozoan trophozoites and cysts. Nonetheless, the use of microscopy in

diagnostic laboratories has several important disadvantages. Some parasite species cannot be differentiated based on microscopy only, while detection of other species may need well trained and experienced technicians. The overall diagnostic sensitivity of microscopy is low and in settings with relatively large numbers of negative results, microscopy can be tedious with relatively high costs for each detected case. Some alternatives for microscopic detection of intestinal parasites have been developed. Those based on parasite antigen detection in stool and antibody detection in serum are sensitive and clinically relevant for diagnosing specific infections but still have their limitations. More promising developments in parasite diagnostics can be found in the field of molecular parasitology. Shortly after the development of polymerase chain reaction (PCR) in 1988 (Saiki et al., 1988), De Bruijn (De Bruijn, 1988) predicted this technique to become a valuable way of diagnosing parasitic diseases. Recently, a range of DNA based methods for the detection of intestinal parasites has been described and postulations have been made on the tremendous impact of the implementation of automated DNA isolation and combination of multiplex real-time PCR assays for the detection of parasites, viruses, and bacteria on the differential laboratory diagnosis of diarrhoeal diseases (Morgan & Thompson, 1998a; Mackay, 2004; Verweij, 2004; Monis et al., 2005; Espy et al., 2006). In this thesis, strategies for the most effective application of these techniques in patient care and epidemiology are evaluated and additional assays for parasitic targets that are currently missing have been developed and validated.

CHALLENGES OF MICROSCOPIC DIAGNOSIS

In Western European laboratories the diagnosis of intestinal parasites by microscopy is facing several important methodological issues that concern the reliability of the analysis. Because so many parasite species are present in faecal specimens in low quantities only, infections are often missed in a direct smear examination. Therefore, concentration methods which increase the recovery of protozoa cysts and helminth eggs, such as the formol-ether sedimentation method, have become a routine procedure in clinical diagnostic laboratories (Ridley & Hawgood, 1956; Allen & Ridley, 1970; anonymous, 1977; Polderman, 2004). For further improvement of the sensitivity, it has been recommended to perform stool examination on samples obtained on different days at intervals of 2 to 3 days (Nazer et al., 1993; Branda et al., 2006). On the other hand sensitivity can be affected if microscopy can not be performed within one hour after defecation, due to the rapid disintegration of trophozoites. To overcome this problem a preservative, such as sodium acetate acetic acid formalin (SAF), can be added to the stool sample immediately after production. This increases the chance of detecting protozoan parasites, in particular *Dientamoeba fragilis* (Mank et al., 1995b).

During the last decade an increasing number of laboratories in The Netherlands have implemented a Triple Faeces Test (TFT)-protocol in order to solve some of these sensitivity limiting factors. In this TFT procedure the patient collects faeces on three consecutive days in two tubes already containing SAF (Van Gool et al., 2003). The SAF preserved specimens are in particular suitable for the detection of trophozoites and for making permanent stains (e.g. with chlorazol black dye (CB) or Iron Haematoxylin-Kinyoun (IHK)). The unpreserved specimen is used for formol-ether concentration of protozoan cysts and helminth eggs, and the detection of *Strongyloides stercoralis* larvae. Although the TFT-protocol has been received as a valuable adaptation of conventional microscopic analysis, one has to consider that the TFT procedure is a more time-consuming approach and needs specific training of the microscopist. Studies in which the recovery of intestinal parasites have been compared using TFT protocol versus the conventional diagnostic method (ether-sedimentation of one fresh stool sample) are limited. Data of two such studies showed that the majority of additional gain in the TFT-protocol are non-pathogens while for the pathogens the largest profit goes mainly to *Giardia lamblia* and *Dientamoeba fragilis* (although the clinical relevance of the latter is still disputed) (Van Gool et al., 2003; Vandenberg et al., 2006a). For some intestinal parasite species, neither examination of a formal-ether concentrated fresh stool sample nor a TFT-procedure is sufficient. Additional procedures are required for the specific detection of *Strongyloides stercoralis* (e.g. Baermann method) and microsporidia (e.g. optical white staining). For the detection of coccidia an acid fast staining procedure is essential, which may be included if the IHK permanent staining is used, but is not covered in the CB staining. Examination with UV fluorescence microscopy is needed for the detection of *Cyclospora cayetanensis* (Polderman, 2004).

Not surprisingly, all these methodological issues are hardly subjects of discussion in laboratories in less developed countries. The application of diagnostic tools does not only depend on the stated objectives of a clinical diagnostic laboratory, but also on the practical limitations when working under basic conditions. More extensive procedures for parasite detection, such as complicated staining techniques or the use of fluorescent light microscopy or even a centrifuge, are often not at hand. Standard routine diagnostic stool examination in resource-poor settings is generally limited to direct faecal smear examination. Additional diagnostic methods for the detection of a specific parasite species are applied mostly when diagnosis is part of major intervention studies, such as the Kato method for monitoring *Schistosoma* infections in high endemic areas (Katz et al., 1972).

Last but not least, the last methodological aspect affecting the reliability of microscopy is the performance of the microscopist. Proper identification of the parasite depends highly on her or his skills and experience. Keeping up with high

standards and performance in clinical diagnostic laboratories requires continued in service-training of the technicians accompanied by indispensable internal- and external proficiency testing (Bartlett et al., 1994).

There are also factors, which are beyond the techniques used and the performance of the technicians, explaining why parasites may remain undetected. As stated, certain parasite species such as *S. stercoralis* or *C. cayetanensis* need specific diagnostic procedures in order to be detected microscopically in the most sensitive and specific way. Because many of these procedures are time-consuming and laborious, they are often not included in routine stool examination, certainly not when the parasite is not very common in the region. Instead many laboratories perform these additional procedures only for individual patients, either based on a special request from the health care provider or justified by information provided about the patient such as travel history or immune disorders. Thus, depending on the stringency of the selection criteria used and the, often limited, information provided by the health care taker, a relevant proportion of parasite infections may be left undiscovered (Whitty et al., 2000; Jones et al., 2004).

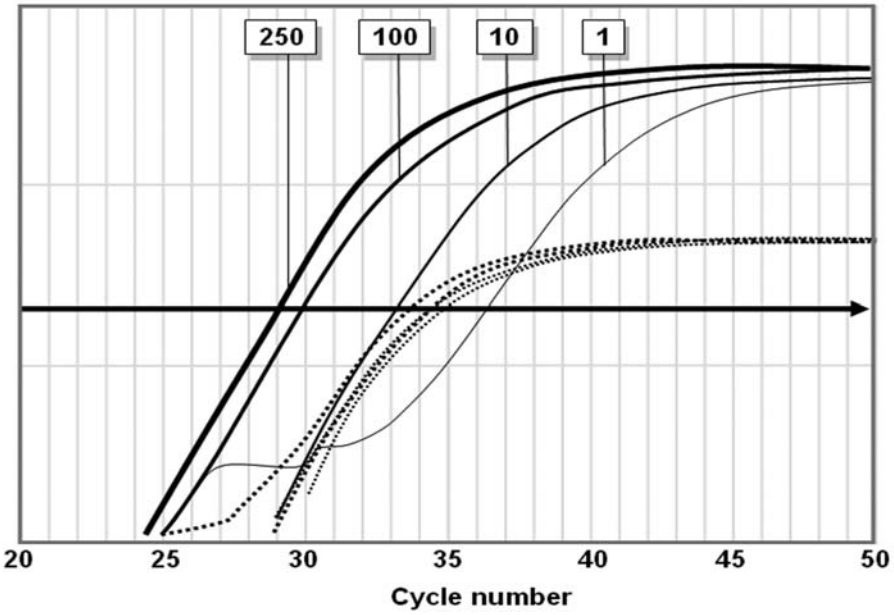
ALTERNATIVE TECHNIQUES

A more provoking approach would appear to be the design of a diagnostic strategy where clusters of patients with some general characteristics are routinely screened for a selected number of parasite species. Such an approach can have a major impact on cost-efficiency in diagnostic laboratories with less demand on highly specialized human resources and labour-intensive microscopy. An initial step in this direction has been the development of immunoassay's (e.g., direct immunofluorescence assays [IFA] or enzyme immuno assays [ELISA]) for antigen detection of *Giardia lamblia, Cryptosporidium* and *Entamoeba histolytica,* which give highly reproducible results which are less dependent on the skills of a lab-technician. High sensitivity and specificity was established with the ELISA for *Giardia lamblia* in comparison with microscopy (Mank et al., 1997), but less consistent results were seen with *Cryptosporidium* antigen detection (Doing et al., 1999; Katanik et al., 2001; anonymous, 2004; Weitzel et al., 2006) whereas the sensitivity and specificity of *Entamoeba histolytica* stool antigen detection assays decreases substantially unless samples are examined or frozen shortly after production (Tanyuksel & Petri, 2003; Visser et al., 2006). For the diagnosis of schistosomiasis in epidemiological research, circulating anodic antigen and circulating cathodic antigen (CCA) detection in serum and urine antibodies have been proposed as an alternative method (Van Lieshout, 1996). In an effort to improve field based diagnosis of schistosomiasi, antigen capture dipsticks that detects CCA in urine have been developed (Van Dam et al., 2004; Stothard et al., 2006). Sensitivity and specificity of the antigen capture

dipsticks still needs to be improved for low endemic areas and so far the test has no proven value for the diagnosis of *Schistosoma haematobium* infections (Stothard et al., 2006; Legesse & Erko, 2008). Nevertheless, CCA dipsticks have the important advantage to be able to semi-quantify *Schistosoma mansoni* infections and not having to rely on a well equipped laboratory.

During the last years, remarkable progress has been made in developing diagnostic methods that are based on the Polymerase Chain Reaction (PCR) technique, particularly since major drawbacks with this technique were readdressed. Initially, DNA isolation from faecal specimens was hindered by time-consuming methods and the presence of inhibitory substances in such samples. PCR was also known as a laborious and expensive technique and inefficient when large number of samples had to be screened for multiple diagnostic targets. Conventional methods of DNA amplification of large number of samples poses a risk to cross-contamination. However, newly developed DNA isolation- and PCR-methods have greatly reduced these obstacles. DNA isolation from stool can be processed in a semi- or fully-automated system with reduced chance of cross-contamination, while removing most of the inhibitory components (Verweij, 2004). After isolation, specific DNA can be amplified and visualized with real-time PCR in closed tubes using fluorescent molecules, which minimizes the risk of cross contamination. Hence, the PCR system simultaneously amplifies and determines the level of amplified DNA products (figure 1.1). Oligonucleotides have been combined with various fluorescent labels that emit light at different wavelengths. In this way, the real-time PCR system has the potential to detect several targets simultaneously in a multiplex real-time PCR assay. An important advantage of a multiplex assay is the reduction in reagent costs and labour time. Furthermore, one of the targets can be assigned to an internal control that can demonstrate inhibitory factors during the amplification process. Several real-time PCR assays have already been developed for clinical diagnosis but because only a limited number of targets can be assigned to one assay, the targets of choice need careful evaluation (Mackay, 2004; Espy et al., 2006).

Figure 1.1. Detection of parasite DNA from faecal suspension containing 1, 10, 100 or 250 oocysts of a parasite. The continuous curved lines show increased fluorescent signal of the parasite specific probes and the dotted curved lines are from corresponding internal controls. Samples with higher concentration of initial DNA templates will cross the fluorescent threshold (horizontal arrow) at a lower amplification cycle number.



As mentioned before, in developed countries parasitic infections are limited to only a few species and some of those can be associated with specific clinical conditions or patient's background. Patient characteristics could therefore be used as a prognostic tool for the design of a new diagnostic decision tree by taking into account the clinical and demographic characteristics of the patients, such as age, travel history, immune-status, etc. With a decision-tree strategy, the most advantageous diagnostic panel could be determined for conducting real-time PCR analyses.

PCR has been successfully applied in another area of work, which is epidemiological research in endemic areas. It has been shown that field based diagnosis could be replaced by PCR analysis in a central laboratory (Verweij et al., 2003a). Stool specimens are collected, suspended in alcohol and can then be stored at room temperature for a period of several months. Upon arrival at a laboratory with PCR facilities, samples can be further processed for the detection of parasite infections. Using automated DNA isolation methods and a real-time PCR system, samples can be processed even more rapidly with little chance of contamination. A sensitive high-

throughput system could bring major improvements in following-up, monitoring or evaluating parasite intervention studies (Mabey et al., 2004; Urdea et al., 2006). Furthermore, the (semi-quantitative) numeric outcome of real-time PCR analysis greatly facilitates the processing, interpretation and reporting of collected data (Verweij et al., 2007a).

In the following paragraphs, specific diagnostic techniques and diagnostic challenges for the most important intestinal parasitic infections will be discussed for each species separately. Although *S. mansoni* and *S. haematobium* are in actual fact blood-flukes, one paragraph will elaborate on the detection of *Schistosoma* species in stool-, urine- and genital-specimens.

ENTAMOEBA HISTOLYTICA.

The perception of the worldwide and regional epidemiology of *E. histolytica* infection has changed after the formal acceptance that the organism called *E. histolytica* in fact consists two genetically distinct species, now termed as *Entamoeba histolytica* (the pathogen) and *Entamoeba dispar* (a commensal) (WHO/PAHO/UNESCO, 1997; Stanley, 2003). Prevalence of *E. histolytica* would therefore be grossly overestimated when the non-pathogenic *Entamoeba dispar* is consistently recognized as an *E. histolytica* infection (Kebede et al., 2003). Epidemiological surveys that used techniques for specific *E. histolytica* detection, have reported prevalence as high as 21% in asymptomatic individuals (Gathiram & Jackson, 1987). In countries where adequate measures are taken for human waste disposal and food- and water-safety, cases of *E. histolytica* infections are sporadic as the parasite is transmitted faecal-orally. In developed countries those still at risk are mainly immigrants and travellers returning from high risk areas, as well as their close relatives (Jelinek et al., 1996; Vreden et al., 2000; Edeling et al., 2004; Boggild et al., 2006). Recognition of *E. histolytica* is of major importance in the clinical laboratory because of its potential of developing into a life-threatening disease. *E. histolytica* infection can result in colitis, dysentery and abscess in organs, most often the liver, with an estimated 40.000 – 100.000 deaths annually (Walsh, 1986; Stanley, 2003). Specific detection of *E. histolytica* cannot be achieved with microscopy alone as cysts and (small) trophozoites of *E. histolytica* and *E. dispar* are morphologically indistinguishable. Therefore, additional methods such as ELISA's for *E. histolytica* antigen detection in faeces are employed, although their performance highly depends on the state of the stool samples (Fotedar et al., 2007). Furthermore, an indirect immunofluorescence assay or a enzyme linked immunosorbent assay (ELISA) for the detection of serum antibodies against *E. histolytica* is a highly sensitive and specific tool to establish the diagnosis of invasive amoebiasis or liver abscess (Jackson et al., 1985; Stanley, 2003; Verweij, 2004). Disadvantages with serology on *E. histolytica* / *E. dispar* carriers are

that in early *E. histolytica* infections the test give false-negative results and in those from endemic areas the test could give false-positive results (Stanley, 2003; Visser et al., 2006). Real-time PCR for the detection of both *Entamoeba* species in stool samples has been recognized as a highly valuable alternative and has become the preferred method in patient diagnostics (Blessmann et al., 2002; Verweij et al., 2003a; Visser et al., 2006).

GIARDIA LAMBLIA

Giardia lamblia (synonyms: *G. duodenalis* and *G. intestinalis*), a flagellated intestinal protozoan parasite, is a common cause of gastroenteritis worldwide (Guerrant et al., 1990). Many people with *Giardia* infection remain asymptomatic and therefore it took many years until the parasite was classified as a pathogen (Rendtorff, 1954; Thompson, 2000). Diagnosis of *G. lamblia* is usually performed by microscopic examination of stool samples for the presence of cysts and/or trophozoites. The excretion of the parasite can be highly variable and therefore analyses of multiple stool samples and concentration techniques are recommended to increase sensitivity (Danciger & Lopez, 1975; Marti & Koella, 1993; Mank et al., 1995b; Hiatt et al., 1995; Polderman, 2004). Other suggested procedures for the detection of *G. lamblia* are concentration techniques and examination of freshly preserved stool samples for the recovery of vegetative stages (Mank et al., 1995b; Polderman, 2004).

The alternatives for microscopic diagnosis of *G. lamblia* include immunoassays for direct immunofluorescence (DFA) and *G. lamblia* antigen detection (Garcia & Shimizu, 1997; Aldeen et al., 1998; Maraha & Buiting, 2000). Compared to microscopy, immunoassay tests have shown increased sensitivity with reduced number of samples required for examination (Mank et al., 1997; Mank & Zaat, 2001) but still multiple stool sample analysis is needed for optimal sensitivity (Hanson & Cartwright, 2001). The second diagnostic alternative is *G. lamblia* DNA detection in stool by real-time PCR, which showed a higher sensitivity than microscopy and immunoassay analyses and can further reduce the required number of stool samples for analysis (Verweij et al., 2003b).

G. lamblia has also been extensively studied with DNA-based methods for classification of subgroups. From humans and various animals, *G. lamblia* strains have been isolated and characterised as different genotypes. The genotypes that can infect humans are clustered in assemblage A and B (Monis et al., 1996; Homan et al., 1998) and several studies have associated assemblages with differences in clinical symptoms. The results of these studies are difficult to compare and sometimes are even conflicting. This subject will be further discussed in **chapters 5** and **11**.

CRYPTOSPORIDIUM HOMINIS / C. PARVUM

Cryptosporidium spp. are coccidian protozoan parasites that infect various vertebrate and invertebrate hosts. At least seven *Cryptosporidium* species have been associated with gastro-intestinal disease in humans: *C. hominis, C. parvum, C. meleagridis, C. felis, C. canis, C. suis* and *C. muris* (Xiao & Ryan, 2004; Caccio et al., 2005). Of these, *C. hominis* and *C. parvum* are the two species found most often in humans. Infections with other *Cryptosporidium* species are sporadic and are associated with a deficient immune system (Pieniazek et al., 1999; Xiao et al., 2000; Gatei et al., 2002). Among immunocompromised individuals, especially those living with AIDS, *Cryptosporidium* is recognised as a potentially life-threatening opportunistic parasite and prevalence is often high in areas affected by the HIV/AIDS pandemic (Guerrant, 1997). *C. hominis* / *C. parvum* may show seasonal distribution patterns and has been recognised as the cause of several water-borne outbreaks (Rose et al., 2002; Karanis et al., 2007; Semenza & Nichols, 2007; Wielinga et al., 2007). Last, *C. parvum* is more associated with zoonotic transmission of infected cattle (Huetink et al., 2001; Lake et al., 2007).

Microscopic detection of *Cryptosporidium* infection usually includes a concentration method in combination with a modified acid fast staining. Microscopic examination can be time-consuming and is highly dependent on technical expertise in a clinical laboratory. As an alternative for microscopy, a variety of commercial tests (IFA and ELISA) have been evaluated for *Cryptosporidium* detection in stool specimens, that have the advantages of improved sensitivity and rapid turnover with little hands-on work (Garcia & Shimizu, 1997; Katanik et al., 2001; Weitzel et al., 2006). However, the applicability of copro-antigen tests in diagnostic laboratories needs to be interpreted with some caution because of conflicting reports on the specificity of the antigens and to the sensitivity of immunodetection methods over microscopy (anonymous, 1999; Doing et al., 1999).

Cryptosporidium-detection methods based on PCR has been an important instrument for studying the taxonomy and transmission of the parasite (Laxer et al., 1991; Jiang & Xiao, 2003; Caccio et al., 2005). More recently *Cryptosporidium* PCR assays were also developed with focus on use in routine clinical diagnostics (Morgan et al., 1998; Morgan & Thompson, 1998b; Verweij et al., 2004a).

ISOSPORA BELLI

Infection with the coccidian *Isospora belli* (recently renamed as *Cystoisospora belli* (Barta et al., 2005; Samarasinghe et al., 2008)) is associated with chronic and severe diarrhoea, in particular in persons living with AIDS and in other immunocompromised individuals (Ferreira, 2000; Lewthwaite et al., 2005; Atambay

et al., 2007). Infections are also seen in children and travellers to tropical regions (Okhuysen, 2001; Goodgame, 2003; Jongwutiwes et al., 2007). An *I. belli* infection is usually diagnosed by microscopic detection of the parasite oocysts in stool samples. The oocysts have a thin transparent shell that makes detection of the oocysts in unstained direct smears difficult and additional microscopic, concentration, and/or staining methods are needed to improve sensitivity (Franzen et al., 1996; Lainson & da Silva, 1999; Bialek et al., 2002). The oocysts can be stained with modified Ziehl-Neelson method and also show auto-fluorescence using a microscope with ultra-violet (UV) light source. A nested PCR method with Southern blot hybridization was described by Muller et al. (Müller et al., 2001) as a helpful technique for the detection of very mild *I. belli* infections. However, these are very laborious procedures and therefore, not efficient for use in epidemiological studies or in routine diagnostic laboratories. Instead, a real-time PCR was developed for the specific detection of *I. belli* DNA in faecal samples which will be further discussed in **chapter 6**.

CYCLOSPORA CAYETANENSIS

Cyclospora cayetanensis, a coccidian intestinal protozoon, is reported to be in the USA the cause of several outbreaks of diarrhoeal illness after import of contaminated fruits and vegetables (Herwaldt, 2000). Infections have also been associated with travellers returning from endemic areas, in particular South-East Asia or South-America (Puente et al., 2006; Kansouzidou et al., 2004; Gascon et al., 1995; Blans et al., 2005). The parasite oocysts are easily missed in faecal wet-mount preparations and staining with modified Ziehl-Neelson gives variable results. However, the thin oocysts wall can well be distinguished by auto-fluorescence using UV-microscopy. Real-time PCR detection for *C. cayetanensis* has been described as a highly sensitive and specific alternative for microscopy (Varma et al., 2003; Verweij et al., 2003c).

DIENTAMOEBA FRAGILIS

Dientamoeba fragilis is a gastro-intestinal flagellate with high prevalence worldwide and is still subject to debate whether the parasite can be considered as a cause of gastro-intestinal illness (De Wit et al., 2001b; Okhuysen, 2001; Johnson et al., 2004; Stark et al., 2006; Vandenberg et al., 2006b; Farthing, 2006). Microscopic diagnosis of the parasite is hindered by its quick decomposition and thus relies on the analysis of fresh stool samples or stool samples fixated immediately after production. Despite the improved diagnosis of *D. fragilis* by the introduction of the TFT protocol the clinical value of this procedure still needs to be evaluated as the protozoa are largely present in asymptomatic persons(De Wit et al., 2001c). Johnson (Johnson et al., 2004) described in his review a number of studies incriminating *D. fragilis* as a

legitimate enteric pathogen. Still, despite of the clinical improvement following the elimination of *D. fragilis* from symptomatic patients, it cannot be ruled out that a substantial number of these patients were suffering from a pathogen that remained undetected with the conventional diagnostic methods used. The introduction of highly sensitive molecular diagnostic methods for intestinal parasites, including the recent developed real-time PCR for the diagnosis of *D. fragilis* (Verweij et al., 2007b), will most likely further clarify the importance of routine diagnosis of *D. fragilis.*

MICROSPORIDIA

Microsporidia are ubiquitously present in nature worldwide. It is a diverse group that represents more than 1200 species with a wide variety of hosts. *Enterocytozoon bieneusi* is the most common species known to cause disease in man. The genus *Encephalitozoon* has three species identified as human pathogens: *E. cuniculi, E. hellem* and *E. intestinalis. E. intestinalis* is the second most prevalent species infecting humans. Before onset of the AIDS pandemic, microsporidial infections had only been described in 10 cases (Weber et al., 1994), but by the expansion of the pandemic, microsporidia came increasingly under attention. The parasites have become important opportunistic pathogens causing several clinical syndromes, most often life-threatening diarrhoea and malabsorption. Apart from its occurrence among AIDS patients, microsporidiosis is nowadays increasingly reported in transplant recipients, children, elderly people and travellers (Fournier et al., 1998; Guerard et al., 1999; Lopez-Velez et al., 1999; Müller et al., 2001; Tumwine et al., 2002; Lores et al., 2002; Leelayoova et al., 2005; Samie et al., 2007). Evidence that *E. bieneusi* is also present in asymptomatic carriers has been accumulating over the last decade (Mathis et al., 2005; Nkinin et al., 2007). This suggests that *E. bieneusi* is a very common intestinal parasite, while the severity of the disease is associated with the immune status of the person. It is also possible that the virulence of *E. bieneusi* is related to different genotypes as recent finding indicate that at least some genotypes show host specificity (Liguory et al., 2001; Sulaiman et al., 2003b, 2004). Although progress is made in increasing the repertoire of techniques for microscopic detection of microsporidia (Garcia, 2002), the interpretation of slides can be very difficult because of the small size of the spores and the variability in the quality of the staining. Moreover, light-microscopy does not allow accurate species identification which it is important as *Encephalitozoon intestinalis* can effectively be treated with albendazole (Sobottka et al., 1995), whereas no established treatment is available for *Enterocytozoon bieneusi* (Conteas et al., 2000). Several PCR assays have been developed for the detection of *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis,* although their application in routine diagnosis is still limited (Katzwinkel-Wladarsch et al., 1996; Kock et al., 1997; Franzen & Müller, 1999; Notermans et al.,

2005). In **chapter 7** a multiplex real-time PCR for the simultaneous detection of *Enterocytozoon bieneusi* and *Encephalitozoon* spp. in faecal samples in routine diagnosis is described.

STRONGYLOIDES STERCORALIS

Strongyloides stercoralis is a soil-transmitted helminth and those living or travelling in (sub)tropical regions are at risk for acquiring an infection. The *S. stercoralis* infection can be perpetuated through a low-grade auto-infection cycle for many decades while most individuals remain asymptomatic (Concha et al., 2005). Under certain conditions, however, an asymptomatic *S. stercoralis* infection can transform into a fulminant fatal hyperinfection with mortality rates of up to 87% (Siddiqui & Berk, 2001; Marcos et al., 2008). Development into a hyperinfection is attributed to a decrease in host resistance caused by debilitating disease, malnutrition or immunosupressive drugs, in particular corticosteroids (Keiser & Nutman, 2004; Fardet et al., 2007). Because of the poor prognosis of a hyperinfection, individuals scheduled for immunosupressive therapy are usually screened for *S. stercoralis* infection. In patients with a chronic infection, diagnosis of *S. stercoralis* is known to be problematic as the number of larvae in a stool sample can be very low. Multiple sampling and concentration methods such as Baermann and copro-culture technique are essential to increase the detection rates (Steinmann et al., 2007).

The success of applying immunodiagnostic assays for the detection of specific antibodies depends on the purity of the antigen used, the antibody isotypes selected for measurement, as well as the population on which the assay is applied on (Polderman et al., 1999; Sudarshi et al., 2003; Van Doorn et al., 2007). Recent infections may result in false-negative results, while persons with a history of residency in tropical regions may prove false-positive as many different assay platforms can not distinguish past from current infection. Antibody detection against a crude extract of *S. stercoralis* filariform larvae can also show cross-reactivity with other helminth infections such as filariasis or schistosomiasis. A positive result with serology may therefore be indicative for *S. stercoralis* infection upon which further searches for the parasite may be required (Grove, 1996). In **chapter 4** and in a study from Verweij et al (Verweij et al., 2009) results indicate that a recent developed *S. stercoralis* real-time PCR assay may provide a valuable alternative as a test for diagnostics and for epidemiological studies.

HOOKWORM

Hookworms are parasitic nematodes that can be found in most of the world's tropical and subtropical countries. The adult worms live in the jejunum and

duodenum, attached to the mucosa and submucosa. The two most important species infecting man are *Ancylostoma duodenale* and *Necator americanus.* Light infections often go unnoticed, while heavy infections can cause hypochrome anaemia; those most at risk are children and pregnant women (Bethony et al., 2006; Calis et al., 2008). Hookworm infections are also occasionally found among persons returning from endemic areas (Ansart et al., 2005; Bailey et al., 2006). Infections can be diagnosed by the microscopic detection of eggs in direct smears or by the examination of concentrated stool samples. Based on the egg morphology, species differentiation is not possible. For the morphological species identification, a copro-culture technique is required to allow eggs to develop and hatch to release the filariform larvae that carry species-specific characteristics. Nevertheless, reliable identification requires time and skilled personnel. Although the differentiation has no clinical importance, it may be a valuable element in epidemiological studies. A recent developed multiplex real-time PCR assay can therefore be regarded as a highly valuable alternative for specification and also semi-quantitative detection of both hookworm species (Verweij et al., 2007a).

SCHISTOSOMA HAEMATOBIUM / S. MANSONI

Schistosoma spp are blood dwelling fluke worms causing schistosomiasis or bilharzia (Gryseels et al., 2006). Worldwide an estimated 200 million people are infected with an approximately 85% of those living on the African continent (Chitsulo et al., 2000). The two most common species infecting humans are *Schistosoma mansoni* and *Schistosoma haematobium.* In schistosomiasis, the pathology is largely determined by the deposition of eggs in the tissues and the extent of granulomatous reactions and fibrosis in the affected organs. Most studies on the pathology of *Schistosoma* infections have focused on the hepatosplenic, bladder and kidney pathology. More recently, attention is drawn to a neglected, but socially important form of pathology of *S. haematobium* infections: female genital schistosomiasis (Poggensee & Feldmeier, 2001; Talaat et al., 2004; Kjetland et al., 2005). What is more indeed, recent studies suggest that as a result of induced inflammation in the semen-producing pelvic organs and in the uterine cervix, both male- and female genital schistosomiasis might constitute a risk factor for HIV transmission (Feldmeier et al., 1994; Leutscher et al., 2005; Kjetland et al., 2006; Secor & Sundstrom, 2007). In developed countries the diagnosis is mainly focused on travellers returning from endemic areas and a routine diagnostic laboratory usually selects methods with the highest sensitivity, specificity and predictive value which include antibody detection in serum and concentration methods for microscopic egg detection in faeces and urine (Lademann et al., 2000; Van Lieshout et al., 2000; Bottieau et al., 2006, 2007). For studies conducted in endemic areas, important criteria for selection of the

diagnostic technique include cost of equipment and adequacy of a method both for the population studied and the involved field-workers. Urine filtration technique for *S. haematobium* (Peters et al., 1976) and Kato thick smear for *S. mansoni* (Katz et al., 1972) are commonly used in control programs and epidemiological studies. These methods allow quantification by egg counts, which generally correlate with worm burdens and morbidity. However, due to a substantial variation in egg excretion light infections are easily missed and examination of stool or urine needs to be repeated on several days (Engels et al., 1996). The use of CCA dipsticks may provide a valuable alternative technique for field diagnosis of intestinal schistosomiasis, although less so for urinary schistosomiasis (Van Dam et al., 2004; Stothard et al., 2006; Legesse & Erko, 2008). So far, no acceptable method has been found for diagnosing genital schistosomiasis and for measuring the morbidity of the disease (Kjetland et al., 2005).

To date, conventional PCR methods for the detection of *Schistosoma* DNA in human samples have been published (Pontes et al., 2002, 2003; Sandoval et al., 2006). More recently, a real-time PCR using SYBR Green dye for the detection of *S. mansoni* has been published (Gomes et al., 2006). Although high sensitivity on control DNA was achieved, this real-time PCR only detected *S. mansoni* DNA. A more desirable assay would detect both *S. mansoni* and *S. haematobium,* combined with an internal control. Such a multiplex real-time PCR is described in **chapter 9.**

OTHER NEMATODES

Finally, two more soil-transmitted nematodes with worldwide distribution are discussed below. The most prevalent one, *Ascaris lumbricoides,* resides in the small intestines of approximately 1.2 billion people worldwide (Bethony et al., 2006; Lammie et al., 2006). Infection with *A. lumbricoides* is often accompanied by *Trichuris trichiura,* an inhabitant of the large intestines of about 800 million people worldwide (Bethony et al., 2006). Although most infections with these nematodes remain unnoticed by the patient, high worm loads, especially in children, may cause severe complications such as dietary deficiencies and delayed physical and cognitive development (Hotez et al., 2007). To control the high morbidity due to these soil transmitted nematodes, including also hookworms, preventive chemotherapy was endorsed through regular administration of antihelminthic drugs in national control programs (Crompton, 2006). However, a recent review and meta-analysis suggested that the efficacy of available anthelminthic drugs is highly overestimated (Keiser & Utzinger, 2008). Diagnosis of *A. lumbridoides* and *T. trichiura* infections is relatively easy using microscopy for detection of the distinctive eggs in stool samples. A real-time PCR for *A. lumbricoides* targeting the rDNA has been described by Pecson (Pecson et al., 2006) for use in both research and routine diagnosis. The diagnosis of

T. trichiura infection still relies on microscopic examination of stool samples.

THE STRATEGY FOR REAL-TIME PCR IN PARASITE DIAGNOSTICS

Based on the given summary of various diagnostic methods for intestinal parasites, the choice of a certain diagnostic technique clearly depends on the objectives and constraints of the laboratory. Choices can be made on the basis of the number of samples, the diagnostic targets and the accuracy of the tests, whereas restrictions may depend on the costs per detection. For the detection of parasites in areas with limited laboratory facilities, several practical constraints can be added. Depending on the diagnostic strategy, real-time PCR might be a cost-efficient diagnostic tool: different real-time PCR detection panels can be designed specifically targeting the most important parasites for the specific patient populations.

Two most important causes of protozoan diarrhoea worldwide are *Giardia* and *Cryptosporidium* (Caccio et al., 2005). For The Netherlands, prevalence of *Giardia* is estimated to be around 5% in patients with gastroenteritis, followed by *Cryptosporidium* with 2% based on microscopy of a single stool sample (De Wit et al., 2001a). Recently, a multiplex real-time PCR was developed for simultaneous detection of *Entamoeba histolytica, Giardia lamblia* and *Cryptosporidium parvum* / *C. hominis* (HGC-PCR) (Verweij et al., 2004a). Although infection with *E. histolytica* is rare in the Dutch population, the target was included in the HGC-PCR because of its potential of developing into a life-threatening disease. In **chapter 2** the HGC-PCR is evaluated for use in routine diagnostic laboratories as an alternative to the diagnosis procedure normally preferred. This study evaluates whether the molecular approach could eliminate unnecessary testing by directing the initial examination to parasites that are most prevalent in the target population, unless clinical considerations dictate otherwise. For the same patient group, results of *Cryptosporidium* detection with the HGC-PCR are discussed in more detail in **chapter 3**. In contrast to local patients attending their general practitioner, returned travellers are at risk of harbouring a larger variety of intestinal parasites. For this group of individuals the most effective diagnostic approach is investigated in **chapter 4** by comparing diagnostic care as usual (i.e. microscopy and copro-antigen detection) with and an adapted real-time PCR panel. Also patient characteristics and clinical data were recorded to develop a diagnostic strategy for implementation of molecular diagnostics methods in the routine diagnosis of intestinal parasitic infection in returning travellers and immigrants. With the detailed clinical and demographic data available, the assemblages of *G. lamblia* are investigated in **chapter 5** for their association with the presence and severity of gastro-intestinal symptoms. *Isospora belli* is a protozoan intestinal parasite which is responsible for diarrhoea with morbidity directly related to the degree of immunodepression. **Chapter 6** describes

the development of a real-time PCR assay for the detection of *I. belli* in stool samples. The assay also has potential to be part of a PCR panel for the immune-compromised patient group when combined in a panel with a multiplex real-time PCR assay for the detection of *Enterocytozoon bieneusi* and *Encephalytozoon* spp. (**chapter 7**). Although *E. bieneusi* is recognised as an opportunistic parasite of HIV/AIDS patients, the parasite is increasingly described also in transplantation patients, travellers, elderly people and children. To elucidate the dynamics of microsporidia infections in different human populations, **chapter 8** describes several identified genotypes obtained from those living in a region with high HIV prevalence (Malawi) and The Netherlands, comparing immune-competent and patients with different types of acquired immune deficiencies.

Obviously, in low income countries real-time PCR has little value for day-to-day diagnosis of intestinal parasites because of high costs. On the other hand, prices of the necessary equipment and consumables are becoming more attractive and new developments in molecular parasitology have made it feasible to introduce real-time PCR in field research as an alternative for conventional microscopic diagnosis. In the field, research is often conducted under basic conditions and can be very strenuous when good storage facilities and appropriate diagnostic equipment are not available. Proper microscopic analysis also depends on the availability of well-trained and supervised technicians. The option for multiplex real-time PCR system was therefore evaluated in **chapter 9** as a new method for the detection and quantification of *S. mansoni* and *S. haematobium* in stool samples collected in an area endemic for both species. Finally, this molecular diagnostic approach was evaluated as an indicator for clinical manifestations of genital *S. haematobium* infection in rural Zimbabwean women (**chapter 10**).