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Molecular detection of intestinal parasites for clinical diagnosis and epidemiology

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**Molecular Detection of Intestinal Parasites for Clinical
Diagnosis and Epidemiology**

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Cover: a photo of *Enterocytozoon bieneusi* was kindly provided by Eric Brienens.

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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 Leeuwenhoek 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).

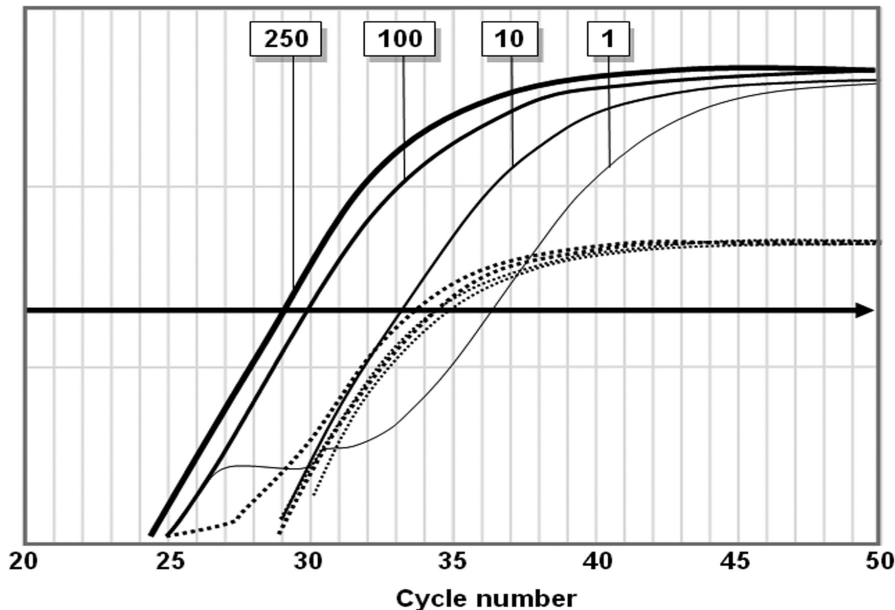
ALTERNATIVE TECHNIQUES

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 immunoassays (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 schistosomiasis, 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).

DIENTAMOEBIA 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 immunosuppressive drugs, in particular corticosteroids (Keiser & Nutman, 2004; Fardet et al., 2007). Because of the poor prognosis of a hyperinfection, individuals scheduled for immunosuppressive 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 HAEMATOBIMUM / 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 anthelmintic drugs is highly overestimated (Keiser & Utzinger, 2008). Diagnosis of *A. lumbricoides* 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**).

CHAPTER 2

DETECTION OF DIARRHOEA-CAUSING PROTOZOA IN GENERAL PRACTICE PATIENTS IN THE NETHERLANDS BY MULTIPLEX REAL-TIME PCR

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ABSTRACT

The diagnostic value of a multiplex real-time PCR for the detection of *Entamoeba histolytica*, *Giardia lamblia* and *Cryptosporidium parvum*/*Cryptosporidium hominis* was evaluated by comparing the PCR results obtained with those of routinely performed microscopy of faecal samples from patients consulting their general practitioner (GP) because of gastrointestinal complaints. Analysis of 722 faecal DNA samples revealed that the prevalence of *G. lamblia* was 9.3% according to PCR, as compared to 5.7% by microscopy. The number of infections detected was more than double in children of school age. Furthermore, *G. lamblia* infection was detected in 15 (6.6%) of 228 faecal samples submitted to the laboratory for bacterial culture only. *C. parvum*/*C. hominis* infections were not diagnosed by routine procedures, but DNA from these organisms was detected in 4.3% of 950 DNA samples. A strong association with age was noted, with *Cryptosporidium* being detected in 21.8% of 110 children aged <5 years. *C. hominis* was the most prevalent species. *E. histolytica* was not detected in this study population. Analysis of microscopy data revealed that the number of additional parasites missed by PCR was small. Overall, the study demonstrated that a multiplex real-time PCR approach is a feasible diagnostic alternative in the clinical laboratory for the detection of parasitic infections in patients consulting GPs because of gastrointestinal symptoms.

INTRODUCTION

Diarrhoea is a major health problem worldwide, killing c. 3–4 million individuals each year. Those most affected by diarrhoea are children and immunocompromised individuals living in developing countries. Although the mortality rate from diarrhoea in developed countries has fallen considerably, morbidity remains high (Kosek et al., 2003). In most cases, the aetiologies of diarrhoea are related to viruses, bacteria and parasites. In The Netherlands, there are c. 4.5 million cases of gastroenteritis annually (De Wit et al., 2001a). The intestinal parasite with the highest prevalence is *Giardia lamblia*, followed by *Cryptosporidium parvum*/*Cryptosporidium hominis* (De Wit et al., 2001c). Infection with *Entamoeba histolytica* is rare, but its high morbidity and, in particular, mortality make accurate diagnosis crucial. Classically, diagnosis of *Giardia*, *Cryptosporidium* and *E. histolytica* infections is achieved by microscopical examination of faecal samples. However, microscopy has several important disadvantages: (i) correct identification depends greatly on the experience and skills of the microscopist; (ii) sensitivity is low, and therefore examination of multiple samples is needed; (iii) *E. histolytica* cannot be differentiated from the non-pathogenic *Entamoeba dispar* simply on the basis of the morphology of cysts and small trophozoites; and (iv) in settings with relatively large numbers of negative results, e.g., The Netherlands, microscopy can be tedious, with relatively high costs for each case detected.

Although molecular methods such as PCR have proven to be highly sensitive and specific for the detection of *E. histolytica*/*E. dispar*, *G. lamblia* and *C. parvum*/*C. hominis* infections (Morgan & Thompson, 1998b; Ghosh et al., 2000; Blessmann et al., 2002; Verweij et al., 2003a, 2003b), their use in routine diagnostic laboratories is still very limited. The introduction of molecular methods has been hindered by time-consuming methods for the isolation of DNA from faecal specimens and the presence of inhibitory substances in such samples. Furthermore, amplification of DNA was previously laborious and expensive, and cross-contamination among samples was a notorious problem. However, newly developed methods have greatly reduced these obstacles (Verweij et al., 2000; Beld et al., 2000; Espy et al., 2006). Real-time PCR reduces labour time, reagent costs and the risk of cross-contamination, and offers the possibility of detecting multiple targets in a single multiplex reaction. A multiplex real-time PCR has been described for the simultaneous detection of the three most important diarrhoea-causing parasites, i.e., *E. histolytica*, *G. lamblia* and *C. parvum*/*C. hominis*, and has demonstrated high sensitivity and specificity with species-specific DNA controls and a range of well-defined stool samples (Verweij et al., 2004a). However, the role of this assay as a diagnostic tool in a routine clinical laboratory requires further evaluation with

respect to large-scale screening and improved patient diagnosis (Lewthwaite et al., 2005; Espy et al., 2006).

In the present study, the diagnostic results obtained using a multiplex real-time PCR for the detection of *E. histolytica*, *G. lamblia* and *C. parvum/C. hominis* were compared with those obtained by routine microscopy of faecal samples from patients visiting their general practitioner (GP) because of gastrointestinal symptoms.

MATERIAL AND METHODS

FAECAL SPECIMENS

DNA samples (n = 956) were initially obtained as part of a study designed to evaluate the efficacy of molecular diagnosis of *Salmonella enterica* and *Campylobacter jejuni* (Schuurman et al., 2007a), and were selected from c. 1900 faecal samples submitted between June and September 2005 to the Laboratory for Infectious Diseases, Groningen, The Netherlands, for routine bacterial culture and/or microscopical analysis. Faecal specimens from which DNA was extracted were submitted with a request from the GP for bacterial culture for *Salm. enterica*, *Shigella* spp. or *Camp. jejuni*, and contained a sufficient amount of faecal material for all tests. Other specimens received during this period were not subjected to DNA extraction.

All samples originated from patients who had visited their GP during the previous few days and who were suspected of having a gastrointestinal infection. According to the normal practice of the GP concerned, each patient submitted either an unpreserved faecal sample (classical method) or a set of three faecal samples (Triple Faeces Test; TFT) collected on consecutive days; one sample was unpreserved and two samples were preserved with sodium acetate acetic acid formalin (Van Gool et al., 2003). Upon arrival, the consistency of and the presence of mucus and/or blood in the unpreserved sample were recorded by a trained technician.

By definition, all 950 unpreserved faecal samples included for PCR analysis (six faecal samples were excluded because of inhibitory substances in the DNA extracts) were cultured routinely for *Campylobacter* spp., *Salmonella* spp. and/or *Shigella* spp., and in some cases for other pathogens, according to the request made by the GP or the clinical microbiologist. From this group, 722 samples were also examined by microscopy for the presence of parasites, according to the request made by the GP or the clinical microbiologist. For comparison, an additional 913 samples from the same group of 1900 cases who submitted faecal samples for the detection of parasites only were also examined by microscopy.

MICROSCOPY

Unpreserved samples were investigated for ova and cysts by microscopy of iodine-

stained wet-mount preparations of a formalin–ether concentrate (Allen & Ridley, 1970). Sodium acetate acetic acid formalin-preserved samples were first screened by iodine-stained direct smears. Parasite-like structures were confirmed by microscopy of a chlorazol black dye permanent stain preparation (Van Gool et al., 2003). Modified Ziehl–Neelsen staining for the detection of *Cryptosporidium* was only performed if cryptosporidiosis was suspected by the GP.

DNA EXTRACTION

DNA was extracted from faecal suspensions (33–50% w/v) using the semi-automated NucliSens miniMAG instrument (bioMérieux, Boxtel, The Netherlands) in combination with NucliSens Magnetic Extraction Reagents (bioMérieux), according to the manufacturer's instructions. In brief, 100 µL of faecal suspension was added to 2 mL of lysis buffer and incubated at room temperature for 10 min, after which an internal control (Phocin herpes virus-1 (PhHV-1); c. 6000 copies/sample) and 50 µL of magnetic silica particles were added. The mixture was mixed and incubated for 10 min at room temperature. After centrifugation for 2 min at 1500 g, the supernatant was removed by aspiration and the pellet of silica–nucleic acid complexes was resuspended and washed in three washing buffers. Each washing step was conducted for 30 s on step 1 of the miniMAG instrument, with the exception of wash buffer 3 (15 s on step 1), after which the fluid was removed by aspiration. DNA was eluted in 100 µL of elution buffer for 5 min at 60°C on a thermoshaker (Eppendorf, Hamburg, Germany) at 1400 rpm. The extracted DNA was stored at –20°C.

PCR AMPLIFICATION AND DETECTION

The sample population was analysed by real-time PCR without reference to the initial microscopy results. Amplification and detection of *E. histolytica*, *C. parvum*/*C. hominis* and *G. lamblia* DNA, as well as the PhHV-1 internal control DNA, were performed on all samples using a multiplex real-time PCR, essentially as described previously (Verweij et al., 2004a), but with minor modifications.

In brief, amplification reactions were performed in 25-µL volumes containing PCR buffer (Hotstar mastermix; Qiagen, Venlo, The Netherlands), 5 mM MgCl₂, 2.5 µg of bovine serum albumin (Roche Diagnostics, Almere, The Netherlands), 3.125 pmol each of the *E. histolytica*- and *G. lamblia*-specific primers, 12.5 pmol of the *Cryptosporidium*-specific primer, 1.25 pmol of VIC-labelled MGB-Taqman probe (Applied Biosystems, Warrington, UK) for *E. histolytica*, 2.5 pmol of FAM-labelled double-labelled probe (Biolegio, Nijmegen, The Netherlands) for *G. lamblia*, 2.5 pmol of Texas-red-labelled double-labelled probe for *Cryptosporidium*, and 5 µL of template DNA. The PhHV-1-specific primers and probe set consisted of 3.75 pmol of each PhHV-1-specific primer and 2.5 pmol of Cy5-labelled double-labelled probe.

Amplification comprised 15 min at 95°C, followed by 50 cycles of 15 s at 95°C and 30

s at 60°C. Amplification, detection and data analysis were performed using the I-cycler Real-Time PCR System and v.3.1.7050 software (Bio-Rad, Hercules, CA, USA).

Faecal DNA samples were considered to contain inhibitors if the PhHV-1 internal control was not detected, or if the expected cycle threshold (Ct) value of 32 cycles was increased by more than five cycles. Because of inhibition, six samples were excluded from the parasite analysis.

Ct values obtained for *G. lamblia*, *E. histolytica* or *Cryptosporidium* amplification are considered less reproducible at >36 cycles. Therefore, the real-time PCR was repeated for 17 faecal samples with an initial Ct value of 36.4–47.4 (median 40.7). For 12 samples, amplification was detected with comparable Ct values (range 33.0–39.1, median 38.0), while five samples showed no DNA amplification and were therefore considered to be negative.

The primers described by Morgan *et al.* (Morgan *et al.*, 1997) were used to differentiate between *C. hominis* and *C. parvum* in DNA samples positive for *Cryptosporidium* DNA. PCR was performed in 25-μL volumes containing PCR buffer, 5 mM MgCl₂, 2.5 μg of bovine serum albumin, 12.5 pmol of forward primer (021F) annealing to both *C. hominis* and *C. parvum*, 6.25 pmol of *C. hominis*-specific reverse primer (CP-HR) and 6.25 pmol of *C. parvum*-specific reverse primer (CP-CR). Amplification comprised 15 min at 95°C, followed by 45 cycles of 30 s at 94°C, 30 s at 58°C and 30 s at 72°C, with a final extension for 5 min at 72°C. Amplification products (411 bp for *C. hominis* and 312 bp for *C. parvum*) were detected following electrophoresis in agarose 2% w/v gels stained with ethidium bromide.

DATA ANALYSIS

Statistical analyses were performed using SPSS v.11.0.1 (SPSS Inc., Chicago, IL, USA). Proportions were compared among groups, and ORs and 95% CIs were calculated. Continuous variables were described by range and median values, and were compared among groups by the Mann–Whitney rank sum test, with *p* < 0.05 considered to be statistically significant.

RESULTS

STUDY GROUP

Table 2.1 summarises the patient characteristics of the 950 cases included for detection of intestinal protozoa by real-time PCR, and the 913 cases examined for parasites only by routine microscopy. The age range of the patients was 0–95 years (median 33 years). The group examined for parasites by PCR contained significantly fewer children aged <15 years as compared to the microscopy group (18.9% vs. 36.8%; OR 0.40, 95% CI 0.33–0.50).

Information concerning travel history was supplied by the GP for 639 (44.3%) patients, including 336 subjects for whom 'no travel history' was specifically noted. Eighty-four patients had travelled in areas considered to be low-risk areas for intestinal parasite infections (western Europe, North America and Australia), while 186 had travelled in areas considered to be high-risk areas (Africa, South America and Asia, including Turkey). No increased frequency of travel was reported for the subjects examined for parasites by microscopy only (table 1). For 33 subjects (age 0–6 years, median 1 year), faecal specimens were sent to the laboratory for routine faecal examination as part of an adoption protocol. The countries of origin of these children were often not reported by the GP.

Information concerning the gastrointestinal symptoms of the patients was supplied by the GP for 65.8% of 950 cases tested by PCR. In most (88%) cases, diarrhoea was mentioned; other symptoms mentioned were watery (11.0%), bloody (5.9%) or slimy (4.8%) faeces. According to the laboratory description, most (90.4%) unpreserved faecal samples were unformed, and the number of watery (3.9%), bloody (1.7%) or slimy (0.8%) samples was limited. Examination for *Cryptosporidium* was specifically requested by the GP for seven patients.

Table 2.1. Characteristics and outcome of faecal examination for parasites in 1863 patients who visited their general practitioner because of gastrointestinal complaints.

	Patient samples examined by		
	PCR and microscopy (<i>n</i> = 722)	PCR without microscopy (<i>n</i> = 228)	Microscopy without PCR (<i>n</i> = 913)
Gender, % male	41.8	41.2	43.4
Age range, years (median)	0–95 (36.0)	0–92 (41.5)	0–92 (27.0)
Travel history, <i>n</i> (%)			
Unknown	578 (80.1)	203 (89.0)	443 (48.5)
None	7 (1.0)	3 (1.3)	326 (35.7)
Low-risk areas	49 (6.8)	6 (2.6)	29 (3.2)
High-risk areas	88 (12.2)	5 (2.2)	93 (10.2)
Adopted child	0	11 (4.8)	22 (2.4)
Microscopy, <i>n</i> (%)			
TFT procedure	247 (34.2)	ND	637 (69.8)
Classical procedure	475 (65.8)	ND	276 (30.2)
<i>Giardia lamblia</i>	41 (5.7)	ND	44 (4.8)
<i>Cryptosporidium</i> spp.	0	ND	1 (0.1)
<i>Entamoeba histolytica</i> / <i>E. dispar</i>	0	ND	1 (0.1)
<i>Blastocystis hominis</i>	21 (2.9)	ND	70 (7.7)
<i>Dientamoeba fragilis</i>	8 (1.1)	ND	17 (1.9)
<i>Endolimax nana</i>	4 (0.6)	ND	14 (1.5)
<i>Entamoeba coli</i>	3 (0.4)	ND	20 (2.2)
<i>Iodamoeba butschlii</i>	0	ND	3 (0.3)
<i>Hymenolepis nana</i>	0	ND	1 (0.1)
Real-time PCR, <i>n</i> (%)			
<i>Giardia lamblia</i>	67 (9.3)	15 (6.6)	ND
<i>Cryptosporidium parvum</i> / <i>C. hominis</i>	36 (5.0)	5 (2.2)	ND
<i>Entamoeba histolytica</i>	0	0	ND

ND, not done; TFT, Triple Faeces Test.

MICROBIOLOGY

In 127 (13.4%) of 950 cases, a non-parasite faecal pathogen was demonstrated. Routine testing revealed *Campylobacter* spp. (n = 90), *Salmonella* spp. (n = 29), *Shigella* spp. (n = 2), *Yersinia* spp. (n = 3) and *Clostridium difficile* (n = 3). Table 1 summarises the results of routine microscopy for faecal parasites. In total, 1635 subjects were examined, either by classical microscopy (n = 751) or by TFT (n = 884). Although significantly fewer TFT procedures were performed in the group analysed for parasites by PCR (34.2% vs. 69.8%; OR 0.23, 95% CI 0.18–0.28), the frequencies of detection of parasites by microscopy in both groups were comparable, with the exception of *Blastocystis hominis* (2.9% vs. 7.7%; OR 2.77, 95% CI 1.68–4.56) and *Entamoeba coli* (0.4% vs. 2.2%; OR 5.36, 95% CI 1.60–18.14). *G. lamblia* was the most common parasite detected (n = 85; 5.2%), either by TFT (n = 48, 5.4%) or by classical microscopy (n = 37, 4.9%; OR 0.90, 95% CI 0.58–1.40). TFT also revealed 25 (2.8%) *Dientamoeba fragilis* infections. Except for one sample with *Hymenolepis nana*, no helminths were seen in any of the faecal samples examined, and only one patient infected with *Cryptosporidium* spp. was detected.

PCR RESULTS

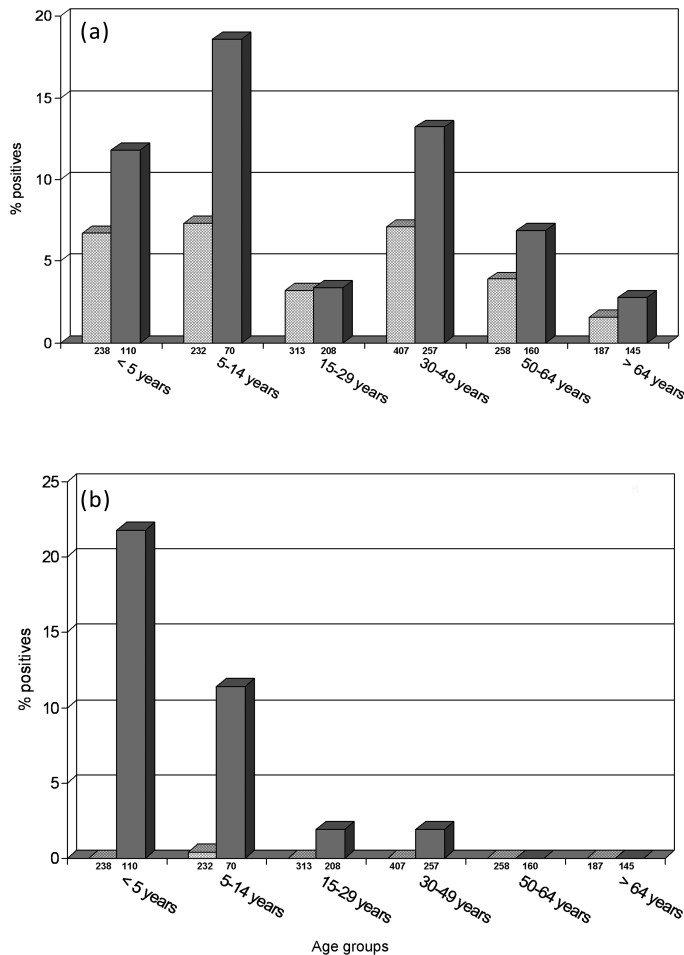
Amplification of the PhHV-1 internal control was, by definition, detected within the correct Ct range for all 950 DNA samples (Ct 29.1–36.9, median 31.7). No *E. histolytica*-specific amplification products were detected.

A *G. lamblia*-specific amplification product was seen for 82 (8.6%) of 950 samples (Ct 21.1–39.1, median 28.6). In the subgroup analysed by both microscopy and PCR, the prevalence of *G. lamblia* increased from 5.7% to 9.3% (table 1). All but one of 41 samples in which *G. lamblia* was detected by microscopy showed a *G. lamblia*-specific amplification product (Ct 21.1–32.8, median 27.5). The discrepant sample (from a male aged 61 years with watery diarrhoea) contained cysts of both *G. lamblia* and *Endolimax nana* when investigated by classical microscopy, with no other pathogens being detected following culture. A *G. lamblia*-specific amplification product was obtained for 16 (3.6%) and 11 (4.8%) of the samples in which *G. lamblia* was not found by classical microscopy or the TFT procedure, respectively. Ct values were significantly higher for those samples in which *G. lamblia* was not detected by microscopy (Ct median 33.0, range 23.2–39.1; $p < 0.001$). Furthermore, 15 (6.5%) samples yielded a *G. lamblia*-specific amplification product, despite the fact that no microscopical examination for intestinal parasites was requested (table 2.1). No significant differences in the Ct values were noted for those samples that were examined microscopically and those that were not.

Compared with microscopy, an increased prevalence of *G. lamblia* infection was revealed by PCR for all age groups, with the exception of patients aged 15–30 years

(figure 1a). The same pattern was seen when the microscopy and PCR data for *G. lamblia* were compared for the 720 subjects analysed by both procedures (data not shown). No associations were seen between *G. lamblia* infection and stool consistency or travel history, with the exception of 11 children who were screened according to the adoption protocol, five (45.5%) of whom yielded a *G. lamblia*-specific amplification product. In comparison, *G. lamblia* infection was indicated in five (22.7%) of 22 children examined by microscopy only.

Figure 2.1. Prevalence of (a) *Giardia lamblia* and (b) *Cryptosporidium parvum* / *Cryptosporidium hominis* in different age groups, detected either by microscopy (hatched bars; n = 1635) or real-time PCR (solid bars; n = 950). The number of cases examined in each category is indicated below each bar.



A *Cryptosporidium*-specific amplification product was obtained from 41 (4.3%) of 950 DNA samples (Ct 24.5–37.9, median 31.0), including two samples in which *G. lamblia* and *Cryptosporidium* were detected simultaneously. *Cryptosporidium* was also detected in five (2.2%) cases for which no microscopical examination was requested by the GP (table 2.1). Infections with *Cryptosporidium* were associated strongly with

the age of the patients, with detectable *Cryptosporidium* DNA in 21.8% of children aged <5 years (figure 2.1b). No association was seen between *Cryptosporidium* infection and travel history. *Cryptosporidium* was detected more often in patients complaining of watery diarrhoea (seven of 72; 9.7%), but this trend was not significant.

Additional PCRs for differentiation of *C. parvum* and *C. hominis* identified 29 samples that contained *C. hominis* and nine samples that contained *C. parvum*; no amplification product was obtained from three samples. *C. parvum* and *C. hominis* were distributed almost equally among the positive samples collected in July, while *C. hominis* was detected exclusively in the samples collected during September (data not shown).

Double infections with parasites and non-parasitic pathogens involved *G. lamblia* and *Campylobacter* spp. (n = 3), and *Cryptosporidium* and *Campylobacter* spp. (n = 1).

DISCUSSION

A multiplex real-time PCR has been described previously for the simultaneous detection of *E. histolytica*, *G. lamblia* and *Cryptosporidium* spp. DNA in faecal samples (Verweij et al., 2004a)[12]. In the present study, the results obtained using this multiplex real-time PCR assay were compared retrospectively with the results obtained by routine microscopy in clinical laboratory practice for patients with diarrhoea who consulted their GP. Faecal DNA samples examined in the present study were isolated initially from a selected number of patients, based on the results of bacterial culture. To evaluate the possibility of selection bias, the microscopy data were compared with those for 913 additional subjects, also suffering from gastrointestinal problems, but with suspected parasitic infections. As expected, the latter group included more children. However, no significant differences in travel history or the number of parasitic infections detected by microscopy were revealed, with the exception of two non-pathogens (*B. hominis* and *E. coli*). In general, pathogenic parasites were detected during routine microscopy in a low number of cases, and with the exception of a single case involving *H. nana*, no helminths were seen. With a detection rate of 5.2% (n = 1635), *G. lamblia* was the most common pathogenic enteric parasite found in patients diagnosed using microscopy. This finding agrees with other studies of *G. lamblia* infection in The Netherlands, including data from a large Dutch case-control study in which *G. lamblia* was detected in 5.4% of patients who consulted their GP for gastroenteritis (De Wit et al., 2001c; Homan & Mank, 2001). Using real-time PCR (n = 950 cases), the rate of detection of *G. lamblia* increased to 8.6%, and the number of infected cases was more than double in children of school age. In addition, very high rates of *G. lamblia* infection were found in adopted foreign children, who had presumably been exposed

in their country of origin (Saiman et al., 2001).

Although no specific request for the diagnosis of *Cryptosporidium* infection was made by the GP, an overall prevalence of 4.3% was detected by PCR, with one in five children aged <5 years being *Cryptosporidium*-positive. Among the infected children, no predisposition towards *C. parvum* or *C. hominis* was noticed. However, a sudden increase in cases positive for *C. hominis* was detected in September 2005, whereas *C. parvum* was detected only in July and August. Seasonal variation in the incidence of cryptosporidiosis related to travel or environmental factors has been suggested (Van Asperen et al., 1996; Caccio et al., 2005; Steffen, 2005), but further investigation is needed to unravel the specific underlying factors explaining this complex epidemiology.

E. histolytica was not detected by real-time PCR in the present study. However, cases of amoebiasis in The Netherlands are very limited, and are always directly or indirectly related to travel in high-risk areas. Nevertheless, accurate diagnosis of *E. histolytica* infection is vital in order to interrupt transmission of the parasite and to avoid any progression into invasive disease. Furthermore, PCR enables specific detection of *E. histolytica*, thereby differentiating this species from the non-pathogenic species *E. dispar*, which is more common and is morphologically identical to *E. histolytica*.

No relationship was found between specific pathogens and the patient's symptoms, as the latter tended to be non-specific and vague, making appropriate diagnostic requests difficult. In faecal samples for which only bacterial culture was requested, real-time PCR revealed 20 parasitic infections. Screening all faecal specimens for parasites in a routine diagnostic laboratory would therefore be appropriate.

The specificity of PCR obviously restricts the number of different pathogens that can be detected, in contrast to the broad range of different parasites that can be detected using microscopy. Nevertheless, microscopy showed limited additional value in this patient population, even with the use of the TFT procedure, as most of the additional parasites detected were non-pathogenic. These included *D. fragilis* and *B. hominis*, two organisms whose pathogenicity is still unresolved. *D. fragilis* is one of the priority candidates for inclusion in an expanded multiplex real-time PCR, as such detection would also provide a valuable tool to further elaborate its possible pathogenicity.

The present study focused on the detection of *E. histolytica*, *G. lamblia* and *C. parvum/C. hominis* in subjects with community-acquired diarrhoea in a developed country. The diagnostic value of this multiplex real-time PCR should also be evaluated in additional patient groups, particularly travellers returning from the tropics and immunocompromised individuals in whom additional intestinal parasites

might be expected. The possibility of combining alternative parasite targets or panels within a multiplex real-time PCR should be investigated, e.g., the detection of *Cyclospora cayetanensis*, *E. histolytica* and *G. lamblia* in travellers, or the detection of microsporidia, *Cryptosporidium* and *Isospora belli* in immunocompromised patients. Used in combination with a similar approach for the detection of diarrhoea-causing viruses and bacteria, this would give a completely new alternative for the laboratory diagnosis of diarrhoeal disease. However, until these approaches have been further evaluated and validated, conventional diagnostic techniques, e.g., culture, microscopy and antigen/antibody detection, will still have a prominent role in the diagnosis of diarrhoeal disease.

In conclusion, the present study revealed that significant numbers of *G. lamblia* and *Cryptosporidium* infections remain undetected by microscopy in patients with gastrointestinal symptoms who consult their GP. Furthermore, the number of additional parasites detected with microscopy was shown to be limited in this population. Therefore, the introduction of real-time PCR for the routine detection of diarrhoea-causing protozoa would improve the diagnostic efficiency of laboratories dealing with faecal samples from this patient group.

CHAPTER 3

CRYPTOSPORIDIOSIS IN DUTCH GENERAL PRACTICE PATIENTS.

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Manuscript in preparation

ABSTRACT

In this study the prevalence of *Cryptosporidium hominis* and *Cryptosporidium parvum* is determined retrospectively in stool samples of 1914 patients attending their general practitioner with gastro-intestinal symptoms in a period of approximately seven months. Microscopic examination aimed at *Cryptosporidium* detection was requested by general practitioners 21 times and found positive in 13 samples. All samples were tested by real-time PCR analysis and revealed 80 cases positive for *C. hominis* / *C. parvum*. Subsequent species specific PCR analysis showed *C. hominis* in 70% of cases and *C. parvum* in 17.5%. In the remaining 12.5% of cases no amplification product was observed. Children under age of 14 years held 70% of all *Cryptosporidium* infections. The highest prevalence was observed the month September. In this month 30% of all children under aged of 5 years were infected, mainly with *C. hominis*. The high *C. hominis* / *C. parvum* prevalence's observed in this survey emphasizes the need for a sensitive high throughput system for *Cryptosporidium* detection in routine diagnostic laboratories.

The protozoan *Cryptosporidium* is an enteric parasite found world wide among a broad variety of hosts (Fayer, 2005). Infection is typically acute and self-limiting, but symptoms can be severe in children and immune compromised persons. In The Netherlands *Cryptosporidium hominis* / *Cryptosporidium parvum* has a prevalence of about 2-3% in cases with gastroenteritis, and about 0.1% in the general population (De Wit et al., 2001a). Detailed epidemiological data on zoonotic potential and transmission routes of *C. hominis* and *C. parvum* in The Netherlands is limited. A recent study of Wielinga *et al.* describes the genetic diversity of *Cryptosporidium* in humans and farm animals from samples gathered over a period of three years at a number of laboratories located throughout The Netherlands (Wielinga et al., 2007). Samples were selected based on microscopic detection of the oocysts. However, in The Netherlands specific diagnostic procedures such as the modified acid-fast staining are not routinely used on each submitted stool sample. Because of the additional work involved, many laboratories perform this staining only if cryptosporidiosis is suspected by the general practitioner (GP). In a previous study 41 (4.3%) *Cryptosporidium* infections were revealed after molecular screening of 950 Dutch patients visiting their GP because of gastro-intestinal complaints (Ten Hove et al., 2007). In the current study an extended group of fecal-DNA samples collected from the same target population was further analysed on the prevalence of *C. hominis* and *C. parvum* in relation to age and the season of sample collection.

DNA of 1914 faecal samples was initially obtained as part of a study designed to evaluate the efficacy of molecular diagnosis for bacterial infections in patients with gastro-intestinal complaints (Schuurman et al., 2007a) and part of samples were also used to compare the molecular diagnosis of intestinal parasitic infections with microscopy (i.e. care as usual) (Ten Hove et al., 2007). The faecal samples from patients were submitted between 27th of June 2005 and 25th of January 2006 to the Laboratory for Infectious Diseases, Groningen, The Netherlands. For all samples the GPs requested bacterial cultures and for 1437 of those also microscopic analysis was requested for diagnosis of intestinal helminthes- and protozoan infections. During the same period, samples were also submitted to the laboratory for the detection of intestinal parasites only. These samples, however, were not included in the initial study (Schuurman et al., 2007a) and were therefore not available for screening with real-time PCR. Faecal DNA extraction was performed as described by Schuurman *et al.* (Schuurman et al., 2007b) and screened with multiplex real-time PCR for presence of *Entamoeba histolytica*, *Giardia lamblia* and *C. parvum* / *C. hominis* (Verweij et al., 2004a; Ten Hove et al., 2007). *C. parvum* / *C. hominis* species were subsequently differentiated with conventional PCR using primer sequences depicted from Morgan *et al* (Morgan et al., 1997) to assess contribution of the zoonotic *C. parvum* infections during late summer when most cases of cryptosporidiosis are expected.

During the period of approximately seven months, *C. hominis* / *C. parvum* infections were detected with real-time PCR among 80 (4.2%) patients with gastro-intestinal complaints (range Ct-values: 24.5 – 42.3; median: 32.1). During the study period microscopic examination for *Cryptosporidium* was specifically requested by GPs 21 times and was found positive in 13 samples. In figure 3.1 the difference in percentages of *C. hominis* / *C. parvum* infections are compared by age groups. The vast majority of *C. hominis* / *C. parvum* infections occur in children under age of 14 years which contain 56 of all 80 detected *C. hominis* / *C. parvum* infections. Species specific PCR showed *C. hominis* in 70% of cases and *C. parvum* in 17.5% of cases. No PCR amplification product was observed in the remaining 12.5% of cases. This can be explained by the high Ct-values (range: 34.2 – 42.3; median 39.0) of these cases which indicate that the number of DNA copies are too low to be detected on ethidium-bromide stained agarose gel. The monthly prevalence of *C. hominis* is presented by a hyper curved shape with its peak in September whereas *C. parvum* prevalence's remains more or less constant (figure 3.2).

Figure 3.1. Distribution of *Cryptosporidium hominis*, *Cryptosporidium parvum* and undetermined *Cryptosporidium* species among patients (n = 1913) classified in age-groups. The age from one patient was not recorded.

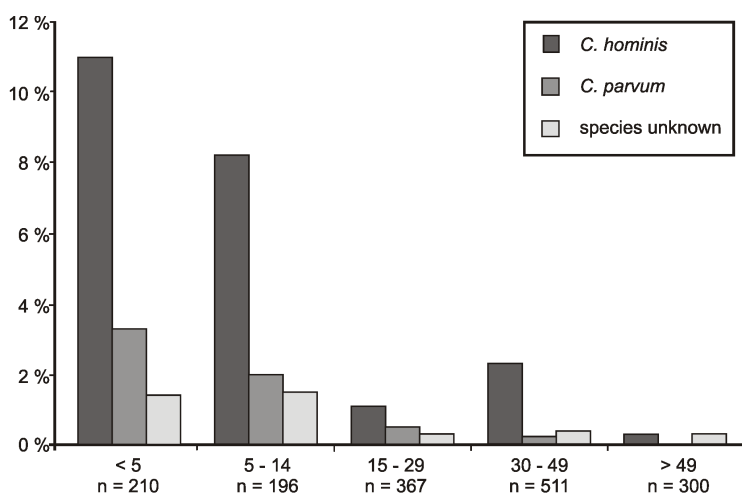
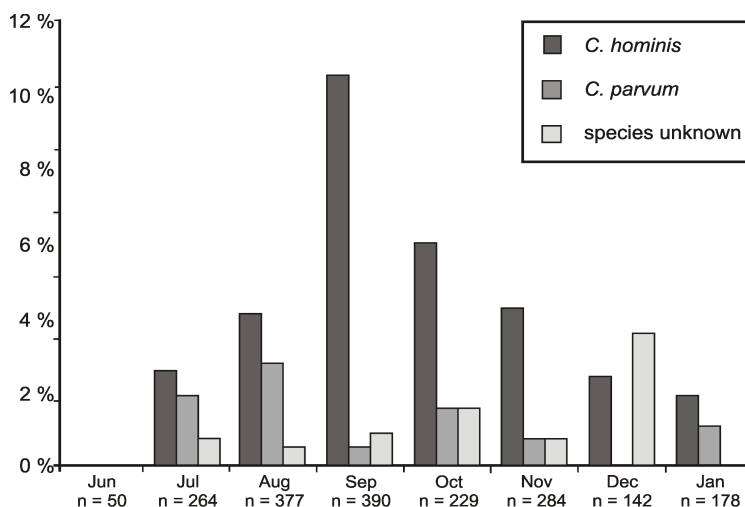


Figure 3.2. Seasonal distribution of *Cryptosporidium hominis*, *Cryptosporidium parvum* and unspecified *Cryptosporidium* species detected in patients (n = 1914).



The outcomes of this study show that current mode of detection greatly underestimates the actual prevalence of *C. hominis* / *C. parvum*. In the month of September, even 30 % of all children under age of five with gastro-intestinal complaints showed to be infected with *C. hominis* / *C. parvum*. The increased prevalence in September was not contributed to increased number of samples from children; throughout the study the monthly percentage of samples originating from children under age of five remained around $11\% \pm 2SD$. Screening for *Cryptosporidium* should be recommended as a standard procedure in stool examination, in particular for children. This is also emphasised by the rate of *C. hominis* / *C. parvum* infections detected in samples that were sent to the laboratory without even a request for parasitological analysis. Of 477 stool samples that came in with a request only for bacteriology, *C. hominis* / *C. parvum* was still detected in 11 (2.3%) cases.

Outbreaks of *C. hominis* infections are often associated with recreational swimming and with contaminated drinking water whereas *C. parvum* is associated more with zoonotic transmission during the kidding period of live stock animals in early spring (McLauchlin et al., 2000). To investigate the role of *C. parvum* in the general *Cryptosporidium* prevalence, molecular screening of patients should continue through spring season with special attention to agricultural activities nearby residential locations (Lake et al., 2007). The relationship between *C. parvum* isolates can be investigated further by combining acquired demographic data with sub-

genotype characterisation of *Cryptosporidium* isolates from stool samples of humans and live-stock animals (Grinberg et al., 2008).

The high *Cryptosporidium* prevalence's observed in this survey emphasizes the need for a sensitive high throughput system for *Cryptosporidium* detection in routine diagnostic laboratories. Implementation of standard molecular screening will reveal *Cryptosporidium* infections more accurately, recognize outbreaks more rapidly and identify sources of contamination more easily.

CHAPTER 4

MOLECULAR DIAGNOSTICS OF INTESTINAL PARASITES IN RETURNING TRAVELLERS.

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ABSTRACT

A new diagnostic strategy was assessed for the routine diagnosis of intestinal parasites in returning travellers and immigrants. Over a period of 13 months, unpreserved stool samples, patient characteristics and clinical data were collected from those attending a travel clinic. Stool samples were analysed on a daily basis by microscopic examination and antigen detection (i.e. care as usual), and compared with a weekly performed multiplex real-time polymerase chain reaction (PCR) analysis on *Entamoeba histolytica*, *Giardia lamblia*, *Cryptosporidium* and *Strongyloides stercoralis*. Microscopy and antigen assays of 2,591 stool samples showed *E. histolytica*, *G. lamblia*, *Cryptosporidium* and *S. stercoralis* in 0.3, 4.7, 0.5 and 0.1% of the cases, respectively. These detection rates were increased using real-time PCR to 0.5, 6.0, 1.3 and 0.8%, respectively. The prevalence of ten additional pathogenic parasite species identified with microscopy was, at most, 0.5%. A pre-selective decision tree based on travel history or gastro-intestinal complaints could not be made. With increased detection rates at a lower workload and the potential to extend with additional parasite targets combined with fully automated DNA isolation, molecular high-throughput screening could eventually replace microscopy to a large extent.

INTRODUCTION

Over the past decennia, outbound tourism showed a worldwide increase and it is expected that this trend will continue in the future (anonymous, 2001). As a consequence, it is likely that a growing number of international travellers will consult a doctor after their return. Gastro-intestinal disorders are one of the main reasons for returning travellers to seek medical advice (Ansart et al., 2005; Freedman et al., 2006). Moreover, several studies have shown that a large proportion of travellers and immigrants from tropical and subtropical countries harbour intestinal pathogens without clear gastro-intestinal problems (Whitty et al., 2000; Saiman et al., 2001; Caruana et al., 2006; Fotedar et al., 2007). Although this emphasises the need for a standard screening procedure for all travellers, the increasing numbers of samples will become a burden for routine diagnostic laboratories, especially during the peak periods of holidaying. Patients and diagnostic laboratories would, therefore, greatly benefit from the implementation of a sensitive high-throughput system for the screening of intestinal parasites.

Intestinal protozoan infections with *Giardia lamblia* and *Cryptosporidium hominis*/*Cryptosporidium parvum* are the main non-viral causes of diarrhoea in industrialised countries (De Wit et al., 2001a) and are even more frequently seen as the cause of gastro-intestinal complaints in returning travellers (Thielman & Guerrant, 1998; Okhuysen, 2001; Freedman et al., 2006). Although quite rare, the early diagnosis of *Entamoeba histolytica* is of vital importance because of the potential invasive character of this protozoan parasite. Intestinal helminth infections in travellers usually do not cause severe clinical complications. One important exception is *Strongyloides stercoralis*. Unlike other helminth infections, *S. stercoralis* is capable of maturing to the infective filariform stage in the intestinal lumen, causing auto-infection through larval penetration of the intestinal mucosa or the perianal skin. Even after decades, these chronic infections can develop into life-threatening hyper infections in immune depressed patients, especially those receiving immunosuppressive therapy with corticosteroids (Concha et al., 2005). Laboratory diagnosis of schistosomiasis, which is frequently diagnosed in travellers, mainly depends on serology rather than on the microscopic detection of ova in stool and urine in this particular population (Bottieau et al., 2007).

Traditionally, the laboratory diagnosis of intestinal protozoan and helminth infections relies on the detection of trophozoites, cysts, eggs and larvae by microscopic stool examination. Although microscopy is considered to be the gold standard, it is labour-intensive and its diagnostic performance critically depends on well-trained microscopists. To improve sensitivity, multiple specimens and concentration procedures, as well as a variety of staining methods, are needed to achieve ample

sensitivity (Morgan & Thompson, 1998a; Branda et al., 2006). Enzyme immunoassays (Katanik et al., 2001; Weitzel et al., 2006) and direct fluorescent-antibody assays (Garcia & Shimizu, 1997) have been accepted as cost-effective alternative diagnostic methods for the detection of *G. lamblia* and *Cryptosporidium* in stools. The specific detection of *E. histolytica* cannot be achieved using microscopy alone, as cysts and (small) trophozoites of *E. histolytica* and non-pathogenic *E. dispar* are morphologically indistinguishable. Therefore, additional methods such as antigen detection or polymerase chain reaction (PCR) have to be employed (Fotedar et al., 2007). The laboratory diagnosis of *S. stercoralis* is known to be problematic: the sensitivity and specificity of immunodiagnostic assays can vary considerably and the number of larvae in a stool sample can be very low. Multiple samples and concentration methods such as Baermann and copro-culture techniques are employed to increase the detection rates (Steinmann et al., 2007).

Although DNA-based methods for a variety of intestinal parasites have shown excellent sensitivity and specificity, until now, the introduction of these methods in daily laboratory practice has been limited. The introduction of real-time PCR combining several targets into one multiplex assay and the implementation of automated DNA-isolation methods offers the possibility of using DNA-based detection techniques in a high-throughput diagnostic approach. In a previous study, it was shown that in patients attending their general practitioner with gastrointestinal problems, only two parasitic pathogens were found in such a population, i.e. *G. lamblia* and *C. hominis/C. parvum* (Ten Hove et al., 2007). The sensitivity of the multiplex real-time PCR proved to be much higher as compared to microscopy in detecting these two parasitic infections. In returning travellers, a larger variety of parasitic infections can be expected. Presently, one of the constraints of multiplex real-time PCR is the restriction in the number of parasitic targets that can be detected simultaneously. Therefore, a careful assessment is needed for the choice of parasitic targets when molecular diagnostic techniques are implemented.

In this prospective study, the performance of real-time PCR for the detection of *E. histolytica*, *G. lamblia*, *C. hominis/C. parvum* and *S. stercoralis* DNA in faeces was compared with current diagnostic tools, which consist of microscopy and antigen detection in stool samples from patients attending a travel clinic. Patient characteristics and clinical data were recorded to define a practical diagnostic strategy for the implementation of molecular methods in the routine laboratory diagnosis of intestinal parasitic infections in returning travellers and immigrants.

MATERIALS AND METHODS

SAMPLE COLLECTION

Stool specimens were collected between April 2005 and May 2006 from outpatients attending the travel clinic of the Institute of Tropical Medicine (ITM) in Antwerp, Belgium. Age, gender, place of birth, travel history and gastrointestinal complaints were actively recorded by the attending physician and included in the database. Patients who were referred to the clinic specifically for HIV/AIDS related issues were not included in this study. A successive sample from the same patient analysed within 30 days of the preceding sample was excluded from the study. Subsequently, each sample was considered to have been obtained from a new patient. The protocol was approved by the ethical committee of the ITM. Patients gave informed consent and the data were rendered anonymous according to the Belgian legislation.

Stool specimens were collected according to the routine procedure of the ITM: patients were asked to fill an empty tube with stool, preferably at the clinic, or otherwise to send it to the diagnostic laboratory of the ITM by regular mail. Aliquots of the samples were stored in the refrigerator until they were sent to the Leiden University Medical Centre (LUMC) for real-time PCR analysis on a weekly transport by regular mail. Molecular diagnostics were performed immediately after the arrival of the samples and the results were generated blinded to the result of the conventional stool examination at the ITM.

MICROSCOPY AND COPRO-ANTIGEN DETECTION.

Microscopic examination for the presence of ova and cysts was performed daily according to the standard routine procedures at the diagnostic laboratory of the ITM by the examination of unstained and iodine-stained direct smears with saline and unstained and iodine-stained wet mounts after formalin-ether concentration (Loughlin & Spitz, 1949). Additionally, carbol-fuchsin staining was performed on formalin-ether concentrates for the detection of coccidian parasites (Heine, 1982). Both pathogenic and non-pathogenic parasites were recorded.

Copro-antigen enzyme-linked immunosorbent assays (ELISAs) for the detection of *Giardia*, *Cryptosporidium* and *E. histolytica*/*E. dispar* (ProSpecT, Remel, Lenexa, Kansas, USA) were performed on all specimens. The *E. histolytica*-specific copro-antigen tests (TechLab, Blacksburg, VA, USA) was performed if microscopic examination revealed *E. histolytica*/*E. dispar* cysts and/or if the *E. histolytica*/*E. dispar* antigen ELISA showed a positive result. In the diagnostic procedure at the ITM before the start of this study, *E. histolytica*/*E. dispar*-positive samples were routinely tested with the *E. histolytica*/*E. dispar* (HD) PCR (Verweij et al., 2003a). As in this study, *E. histolytica* PCR would be performed as part of the

E. histolytica/*G. lamblia*/*Cryptosporidium* (HGC) PCR on all samples; HD PCR was performed only on the microscopy or antigen *E. histolytica*/*E. dispar*-positive samples retrospectively.

In clinically suspected strongyloidiasis cases, Baermann's method was requested to detect *S. stercoralis* larvae (Jones & Abadie, 1954; Pereira Lima & Delgado, 1961). The detection of *Enterobius vermicularis* eggs was performed on demand using the scotch-tape method (Graham, 1941).

DNA ISOLATION

For DNA isolation, 200µl of faeces suspension (approximately 0.5 g/ml PBS containing 2% polyvinylpyrrolidone [PVPP; Sigma]) was heated for 10 min at 100°C. After sodium-dodecyl-sulphate-proteinase K treatment (overnight at 55°C), DNA was isolated with QIAamp Tissue Kit spin columns (QIAgen, Hilden, Germany) (Verweij et al., 2000). In each sample, 10³ plaque-forming units (PFU)/ml phocin herpes virus 1 (PhHV-1) was added within the isolation lysis buffer, to serve as an internal control for the isolation, amplification and detection of the multiplex real-time PCR assays (Niesters, 2002).

PCR AMPLIFICATION AND DETECTION

E. histolytica, *G. lamblia* and *C. hominis*/*C. parvum* multiplex real-time PCR including PhHV-1 as an internal control (HGC PCR) was performed as described previously with some modifications (Verweij et al., 2004a). *S. stercoralis* DNA amplification was performed in a separate assay, also including PhHV-1 as an internal control (Verweij et al., 2009).

Amplification and detection reactions for HGC PCR were performed in a volume of 25µl containing PCR buffer (Hotstar master mix, QIAgen, Venlo, The Netherlands), 5 mM MgCl₂, 2.5µg bovine serum albumin (BSA) (Roche Diagnostics, Almere, The Netherlands), 3.13 pmol of each *E. histolytica*-specific primer, 1.25 pmol VIC-labelled MGB probe for *E. histolytica* (Applied Biosystems, Warrington, UK), 3.13 pmol of each *G. lamblia* primer, 2.5 pmol *G. lamblia*-specific FAM-double-labelled probe (Biolegio, Malden, The Netherlands), 12.5 pmol of each *C. hominis*/*C. parvum*-specific primer, 2.5 pmol of Texas Red double-labelled probe for *C. hominis*/*C. parvum*, 3.75 pmol of each PhHV-1-specific primer, 2.5 pmol of PhHV-1-specific Cy5- double-labelled probe (Niesters, 2002) and 5µl of the DNA sample. Amplification consisted of 15 min at 95°C followed by 50 cycles of 15 s at 95°C, 30 s at 60°C and 30 s at 72°C. Amplification, detection and data analysis for HGC real-time PCR was performed with the i-Cycler real-time detection system (BioRad, Veenendaal, The Netherlands).

The amplification and detection of *S. stercoralis*-specific DNA was performed with 5

pmol of each *S. stercoralis* specific primers, 2.5 pmol of *S. stercoralis*-specific FAM-double-labelled probe (Biolegio), 3.75 pmol of each PhHV-1-specific primer, 2.5 pmol of PhHV-1-specific Cy5-double-labelled probe (Niesters, 2002), PCR buffer (Hotstar master mix, QIAgen), 5 mM MgCl₂, 2.5µg BSA and 5µl of the DNA sample in a final volume of 25µl (Verweij et al., 2009). Amplification consisted of 15 min at 95°C followed by 50 cycles of 15 s at 95°C and 60 s at 60°C. Amplification, detection and data analysis for *S. stercoralis* real-time PCR was performed with the Applied Biosystems 7500 Real-Time PCR System and Sequence Detection Software version 1.2.2.

At the end of the study period, *E. histolytica*/*E. dispar* positive samples with microscopy- and/or copro-antigen detection HD PCR (Verweij et al., 2003a) was performed, retrospectively. Amplification and detection was performed with 3.125 pmol of each *E. histolytica*/*E. dispar*-specific primers, 2.5 pmol of *E. dispar*-specific FAM-labelled MGB probe (Applied Biosystems) (Verweij et al., 2003a), 3.75 pmol of each PhHV-1-specific primer, 2.5 pmol of PhHV-1-specific Cy5-double-labelled probe (Niesters, 2002), PCR buffer (HotStar master mix, QIAgen), 5 mM MgCl₂, 2.5µg BSA and 5µl of the DNA sample in a final volume of 25µl. Amplification consisted of 15 min at 95°C, 30 s at 60°C and 30 s at 72°C. Amplification, detection and data analysis for HD real-time PCR was performed with the i-Cycler real-time detection system.

High cycle threshold values (Ct-values) obtained by real-time PCR are considered to be less reproducible due to very low copy numbers of the specific target. Therefore, the PCR of samples revealing Ct-values above 36 were repeated. Also, the amplification was considered to be hampered by faecal inhibitory factors if the expected Ct-value of 33 in the PhHV-1-specific PCR was increased with more than 3.3 cycles. Samples were recorded negative for the specific target if the positive (high) Ct-value could not be reproduced or were excluded if the amplification remained inhibited.

DATA ANALYSIS

The results of the microscopic examinations, copro-antigen tests and real-time PCR analysis were stored and grouped in an Access database (Microsoft, Redmond, WA, USA). Data included patient's demographic information, travel history and clinical presentation, the laboratory results of the travel clinic in Antwerpen and the real-time PCR results of the laboratory for parasitology in Leiden. Travel destinations were classified according to geographical regions: Central America, South America, Asia, Northern Africa and sub-Saharan Africa were considered as high-risk areas and others (including Europe, United States of America and Australia) as being at a low risk of contracting intestinal infections. World travellers or sailors were also considered to have a high risk of contracting intestinal infections. Analysis was done with SPSS 14.0 (SPSS Inc., Chicago, IL, USA). Continuous variables were described by

the range and median of all positive cases and were compared between groups by the Mann-Whitney rank-sum test. For this purpose, zero values in Ct-values were redefined as 50. Statistical significance was considered at $P < 0.05$. The Chi-square distribution for the risk of acquiring pathogenic intestinal parasites, recorded as high- or low-risk travel destination, or for the presence of gastro-intestinal complaints, was calculated as a proportion of parasite infections detected by any of the used diagnostic techniques.

RESULTS

STUDY GROUP

Over a period of 13 months, 2,709 samples were collected and analysed (Fig.4.1). One hundred and eighteen samples were excluded from the study population as the time interval after the preceding sample of the same patient was less than 30 days. The final number of participants included in this study was 2,591. PhHV internal control was amplified within the correct Ct range in all samples; therefore, no samples were excluded due to inhibition. The travellers' ages varied from newborn to 85 years (median 36) and 55.5% of the patient cohort was of the male gender. Travel to 142 different countries or areas was mentioned, most frequently to sub-Saharan Africa (50.9%). The majority of travellers were born in Europe (73.7%) or on the African continent (19.0%). Gastro-intestinal complaints were mentioned by 897 (34.6%) patients and are listed in more detail in Table 4.1, together with the other patient's characteristics.

Figure. 4.1 Number of all collected stool samples ($n=2,709$) on a weekly basis between April 2005 and May 2006. Sharp increases of sample collections due to holidays are observed in August 2005 and at beginning of January 2006

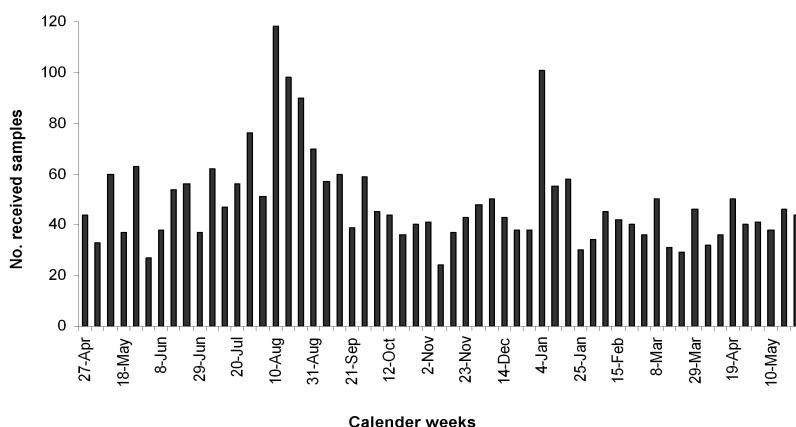


Table 4.1 Epidemiological and clinical characteristics of the study population (*n*=2,591)

Characteristics	<i>n</i> (%)
Patient characteristics	
Male	1,437 (55.5)
Age range, median, years	0–85 (36.0)
Children, age <15 years	161 (6.2)
Elderly, age >59 years	259 (10.0)
Born in Europe	1910 (73.7)
Born in Africa	491 (19.0)
Other/unknown country of birth	190 (7.4)
Last visited region	
Low-risk areas	98 (3.8)
Central America	99 (3.8)
South America	130 (5.0)
Asia	458 (17.7)
North Africa	102 (3.9)
Sub-Saharan Africa	1,318 (50.9)
World travellers	17 (0.7)
Unknown	369 (14.2)
In Belgium since	
<1 month	1,143 (44.1)
1–6 months	579 (22.3)
6–12 months	170 (6.6)
>12 months	476 (18.4)
Unknown	223 (8.6)
Gastro-intestinal complaints	
No gastro-intestinal complaints	1,692 (65.3)
Diarrhea, flatulence, pain and/or nausea	897 (34.6)
Unknown	2 (0.1)
Gastro-intestinal complaints specified	
Diarrhoea	635 (24.5)
Flatulence	380 (14.7)
Cramps/pain	608 (23.5)
Nausea	280 (10.8)
Unknown	2 (0.1)
Duration of complaints	
<2 weeks	334 (12.9)
>2 weeks	563 (21.7)
Stool consistency	
Formed	1,765 (68.1)
Unformed	765 (29.5)
Watery	49 (1.9)
Unknown	12 (0.5)
Bloody	37 (1.4)
Mucous	1 (<0.1)

Table 4.2 Intestinal parasites in the study population ($n=2,591$) as detected with microscopy, copro-antigen test and real-time Polymerase chain reaction (PCR)

Pathogens	Total detected	Microscopy ^a	Antigen	Real-time PCR
<i>E. histolytica/dispar</i>	114	99	90 ^c	
<i>E. histolytica</i>	14	1 ^b	7 ^d	13
<i>G. lamblia</i>	156	95	121	149
<i>Cryptosporidium</i> spp.	33	12	14	31
<i>S. stercoralis</i>	21	3		21
<i>T. trichiura</i>	14	14		
Hookworm	10	10		
<i>A. lumbricoides</i>	8	8		
<i>Trichostrongylus</i> spp.	3	3		
<i>E. vermicularis</i>	1	1		
<i>S. mansoni</i>	11	11		
<i>S. haematobium</i>	1	1		
<i>Taenia</i> sp.	1	1		
<i>C. cayetanensis</i>	4	4		
<i>I. belli</i>	2	2		
Non-pathogens				
<i>E. coli</i>	246	246		
<i>B. hominis</i>	220	220		
<i>E. nana</i>	139	139		
<i>E. hartmanni</i>	59	59		
<i>I. bütschlii</i>	26	26		
<i>C. mesnili</i>	23	23		
<i>S. hominis</i>	6	6		

^a Direct microscopy, after formalin-ether concentration, scotch tape test for *E. vermicularis*, Baermann test for *S. stercoralis*, and carbol-fuchsin staining for *Cryptosporidium* spp.

^b Observed hematophagous trophozoites

^c *E. histolytica/E. dispar* copro-antigen test (ProSpect, Remel, Lenexa, Kansas, USA)

^d *E. histolytica* specific copro-antigen test (TechLab, Blacksburg, Virginia, USA)

DIAGNOSIS OF *ENTAMOEBIA HISTOLYTICA*/*ENTAMOEBIA DISPAR*, *GIARDIA LAMBLIA* AND *CRYPTOSPORIDIUM SPP.*

The results of microscopy, copro-antigen and real-time PCR for all faecal parasites are summarised in Table 4.2. *E. histolytica*/*E. dispar* cysts and trophozoites were detected by microscopy in 99 cases. Fifteen additional cases were detected with *E. histolytica*/*E. dispar* copro-antigen ELISA. The presence of *E. histolytica* was confirmed with the species-specific copro-antigen ELISA in seven cases. In one of these seven samples, trophozoites with ingested red blood cells were seen in the direct smear, representing the only distinctive morphologic features of *E. histolytica*. *E. histolytica*-specific amplification was detected using the HGC PCR in 13 samples, with Ct-values between 20.7 and 38.7 (median 31.3). Retrospective analysis with HD PCR of 114 *E. histolytica*/*E. dispar* microscopy and/or coproantigen-positive samples confirmed 88 samples as *E. dispar* and 14 samples as *E. histolytica*, whereas HD PCR remained negative for both targets in 12 samples.

G. lamblia cysts and/or trophozoites were detected with microscopy in 95 cases and 121 cases revealed a positive result in the *Giardia* antigen ELISA. The *Giardia* antigen ELISA was positive in 28 microscopy-negative cases and did not confirm two microscopy-positive samples. *G. lamblia*-specific amplification was detected in 149 samples with Ct-values between 17.0 and 44.7 (median 29.8).

Cryptosporidium oocysts were detected in 12 carbol-fuchsin-stained samples. The copro-antigen tests confirmed all *Cryptosporidium* microscopy-positive samples and two additional samples were found positive in the *Cryptosporidium* antigen ELISA. *C. hominis*/*C. Parvum* specific amplification was detected in 31 samples with Ct-values between 24.4 and 39.5 (median 35.6).

Ct-values of samples positive with microscopy and/or copro-antigen tests were significantly lower as compared to samples with a negative result in the conventional methods ($P < 0.001$) for both *G. lamblia* and *C. hominis*/*C. parvum* (data not shown).

Discrepancies between real-time HGC PCR and conventional methods were observed in the following cases. *E. histolytica* real-time PCR was negative in one sample in which *E. histolytica*/*E. dispar* cysts were observed with microscopy and both copro-antigen ELISAs tested positive. *Giardia* real-time PCR remained negative in two cases in which cysts were seen in microscopy and in five cases in which only the copro-antigen assay tested positive. *Cryptosporidium* real-time PCR remained negative in two samples in which only the *Cryptosporidium* copro-antigen tested positive. The HGC multiplex real-time PCR detected a total of 49 additional cases which were not detected with microscopy and antigen tests.

STRONGYLOIDES STERCORALIS

Rhabditiform *S. stercoralis* larvae were detected with microscopy on concentrated smears in 3 samples. In one of these samples the number of larvae was exceptionally high and the larvae were also detected in direct smear. Baermann was performed on stool samples from 121 clinically suspected cases and was only found positive in the same sample that was found positive in the direct smear. *S. stercoralis* specific amplification was detected in all 3 microscopy positive samples and in 18 additional samples with Ct-values ranging from 24.5 to 39.5 (median of 33.3).

OTHER PARASITIC INFECTIONS

Microscopy revealed 55 additional pathogenic parasites that were not targeted with real-time PCR. Mixed infections with two pathogenic parasite species were observed in 18 patients (Table 4.3). Non-pathogenic parasites as seen in direct smears and wet mounts after concentration are also summarised in Table 4.2. Travel history, symptoms and intestinal parasites Detected parasitic infections in relation to travel destination and gastro-intestinal complaints are summarised in Table 4.4.

Table 4.3 Observed double infections of pathogenic parasites in stool samples ($n=2,591$), detected by microscopy, copro-antigen detection and/or real-time PCR

Combination of species found	Number of samples
<i>E. histolytica</i> and <i>G. lamblia</i>	1
<i>E. histolytica</i> and <i>Cryptosporidium</i> sp.	1
<i>E. histolytica</i> and Hookworm	1
<i>G. lamblia</i> and <i>Cryptosporidium</i> sp.	4
<i>G. lamblia</i> and <i>S. stercoralis</i>	2
<i>G. lamblia</i> and <i>T. trichiura</i>	2
<i>G. lamblia</i> and <i>Trichostrongylus</i> sp.	1
<i>Cryptosporidium</i> sp and <i>T. trichiura</i>	1
<i>S. stercoralis</i> and <i>A. lumbricoides</i>	1
<i>A. lumbricoides</i> and <i>E. vermicularis</i>	1
<i>A. lumbricoides</i> and hookworm	1
Hookworm and <i>T. trichiura</i>	1
<i>Trichostrongylus</i> sp. and <i>S. mansoni</i>	1

TRAVEL HISTORY, SYMPTOMS AND INTESTINAL PARASITES

Detected parasitic infections in relation to travel destination and gastro-intestinal complaints are summarized in Table 4.4. The majority (69.2%) of the study participants travelled to areas where the exposure risk to intestinal parasites is considered to be high. Among those with high-risk travel destinations, infection rates were not significantly higher for any of the pathogenic parasites compared with those who travelled to low-risk areas. Gastro-intestinal complaints were correlated with *G. lamblia*- and *C. hominis/C. parvum* detection ($P<0.001$), but not with the detection of *E. histolytica*, *S. stercoralis* or other pathogenic parasitic infections.

Table 4.4 Detected intestinal parasites in the study population ($n=2,591$) by real-time PCR, microscopy and/or antigen test listed in numbers and proportion of travel destination or presence of gastro-intestinal (GI) complaints.

	PCR/Microscopy/Ag				Microscopy
	<i>E. histolytica</i>	<i>G. lamblia</i>	<i>Cryptosporidium</i>	<i>S. stercoralis</i>	additional pathogenic parasites
High-risk travel pop. ^a $n=1,793$ (100%)	10 (0.6%)	121 (6.7%)	26 (1.5%)	16 (0.9%)	37 (2.1%)
Low-risk travel pop. ^b $n=429$ (100%)	3 (0.7%)	19 (4.4%)	4 (0.9%)	1 (0.2%)	4 (0.9%)
Destination unknown $n=369$ (100%)	1 (0.3%)	16 (4.3%)	3 (0.8%)	4 (1.1%)	14 (3.8%)
With GI complaints $n=897$ (100%)	7 (0.8%)	76 (8.5%)	25 (2.8%)	4 (0.4%)	18 (2.0%)
No complaints $n=1,694$ (100%) ^c	7 (0.4%)	80 (4.7%)	8 (0.5%)	17 (1.0%)	37 (2.2%) ^d
Total $n=2,591$ (100%)	14 (0.5%)	156 (6.0%)	33 (1.3%)	21 (0.8%)	55 (2.1%)

^a Sub-Saharan Africa, Asia, world travellers

^b Europe, America, Australia, North Africa

^c Two cases with complaints not registered are added to the traveller group without gastro-intestinal complaints

^d Group contains four travelers with double infections

DISCUSSION

The diagnosis of intestinal parasitic infections in returned travellers traditionally relies on time-consuming analyses by experienced microscopists. Nowadays, the increasing number of travellers to a variety of exotic countries calls for new diagnostic approaches for the efficient processing of samples. In this study, multiplex real-time PCR was compared with the routine approach of microscopy and antigen-based methods, focussing on four target parasites, *E. histolytica*, *G. lamblia*, *Cryptosporidium* and *S. stercoralis*, respectively.

The results showed PCR to be more sensitive for the specific detection of *E. histolytica*, *G. lamblia*, *C. hominis*/*C. parvum* and *S. stercoralis*. Only a few unexplained discrepancies between the different detection methods were seen. These were mostly cases in which only the antigen test was positive. In one sample, *E. histolytica*/*E. dispar* cysts were seen with microscopy and both antigen ELISAs showed a positive result; however, *E. histolytica*-specific amplification was not successful in the initial HGC PCR. A positive result was obtained when the HGC real-time PCR was repeated, as well as in the HD real-time PCR. Internal and positive controls in the initial real-time PCR analysis were correct and the reason for the negative outcome of the first real-time PCR analysis remains unclear. This one case is discordant with the proven higher sensitivity of *E. histolytica* real-time PCR compared to stool antigen assays in a non-endemic setting (Visser et al., 2006; Stark et al., 2008).

It is well known that the laboratory diagnosis of *S. stercoralis* requires multiple fresh stool samples using concentration techniques and/or copro-culture in order to improve detection rates (Siddiqui & Berk, 2001). In this study, the Baermann concentration technique was requested by the clinician in case of clinical suspicion, yet many Baermann tests were omitted because, for example, the sample was too old (had been sent by mail). Although the positive results of the *S. stercoralis* real-time PCR was confirmed by microscopy in three cases only, the other positive results were supported by serology ($n=7$), eosinophilia ($n=5$) and/or clinical presentation ($n=4$) in the majority of cases.

In this study population, ten additional pathogenic parasite species detected by microscopy were missed with only four targeted parasite species in the real-time PCR. However, the prevalence of each detected species was only 0.5% at most and the clinical significance of the majority of these parasitic infections is limited. Most of these infections were helminths, of which the eggs could be easily found with microscopy at low magnification of an unstained wet mount of the concentrated sample. Schistosomiasis is probably the most relevant and also probably the most underestimated infection in this study, as the laboratory diagnosis of *Schistosoma*

infections in travellers relies mainly on serology (Bottieau et al., 2006). The implementation of a *Schistosoma* real-time PCR (ten Hove et al., 2008) might be a worthwhile addition to a molecular diagnostic panel; however, its performance in a routine setting still needs further evaluation. Finally, two remaining protozoan infections, *Isospora belli* and *Cyclospora cayetanensis*, are important candidates as additional real-time PCR targets for patients returning from high-risk areas (Varma et al., 2003; Verweij et al., 2003c; Ten Hove et al., 2008).

Designing an efficient diagnostic strategy requires a thorough exploration of possible predictors for parasite exposure in patient groups. A rationale for a specific diagnostic approach in a travel population is less evident compared with a patient group without extensive travel background (Ten Hove et al., 2007). In the travel population of this study, only a minority (34.6%) mentioned gastro-intestinal complaints. Stool examination was also performed as part of a routine screening procedure. The presence of gastro-intestinal complaints was a significant predictor only for the presence of *G. lamblia* and *C. hominis/C. parvum*. In the cases without complaints, however, the prevalence of *G. lamblia* was still higher than any of the other pathogenic parasites in the total population. Furthermore, travel destinations were of little predictive value for the presence of any of the parasitic infections. In conclusion, travel destinations or gastro-intestinal complaints did not provide a diagnostic key towards specific pathogenic parasites in this study. As already seen in other studies, an exception can be made for the diagnosis of schistosomiasis, as cases are mainly found in travellers returning from Africa (Whitty et al., 2000; Bottieau et al., 2006).

The overall low prevalence of intestinal parasitic infections found in this study emphasises the need for a rapid, sensitive and simple screening assay for the most important parasitic infections in all returned travellers, disregarding their travel history and the presence of gastro-intestinal complaints, which agrees with recommendations made in other studies (Whitty et al., 2000; Bailey et al., 2006; Gushulak et al., 2007). The low prevalence of additionally detected parasites raises doubts about the cost benefit of elaborate microscopic analyses of stool samples. An in-depth study on the cost per detected parasite or profit per diagnosis by different technical approaches will further elucidate the most beneficial strategy for a laboratory. For example, the staff utilisation for stool analysis with conventional techniques consists in this study of approximately one full-time equivalent (FTE) compared to 0.3 FTE for stool DNA isolation and real-time PCR analysis. Moreover, a fully automated DNA isolation process and extension of additional molecular targets on the already isolated DNA will have considerable impact on the cost-efficiency of the diagnostic procedures. Already, a growing number of routine diagnostic laboratories are implementing multiplex real-time PCR for the detection of intestinal

microorganisms (Mackay, 2004; Schuurman et al., 2007a; Ten Hove et al., 2007). These standard PCR assays can be extended specifically for the travel population with one or more additional multiplex real-time PCR panels for an overlap of the most important intestinal parasitic infections.

Real-time PCR has proved to be a highly sensitive and specific technique for the detection of the majority of intestinal parasites found in returning travellers presenting at a travel clinic with and without gastro-intestinal complaints. The diagnostic approach for the detection of intestinal parasites in returning travellers in a routine diagnostic laboratory could be limited to real-time PCR for *E. histolytica*, *G. lamblia*, *Cryptosporidium* spp. and *S. stercoralis*. In addition, *Schistosoma* serology should be performed for travellers to Africa (Whitty et al., 2000). This approach could be complemented with additional multiplex PCRs and/or a quick microscopic examination for the presence of helminth eggs. Fully automated procedures and combination with additional targets might replace microscopy in the future to a large extent.

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CHAPTER 5

MOLECULAR EPIDEMIOLOGY OF *GIARDIA LAMBLIA* IN TRAVELLERS.

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Manuscript in preparation

ABSTRACT

Clinical manifestations of *Giardia lamblia* infections are highly variable and can range from asymptomatic to the presence of severe gastro-intestinal complaints. Variety in symptomatology might partly be attributed to a variation in the virulence of *Giardia* assemblages. This study investigated the association of gastro-intestinal symptoms with the intensity of *G. lamblia* infections and with the identified assemblages A and B in a predominant adult travel population. Intensity of *G. lamblia* infections of 132 positive cases showed correlation with their complaints categorized by its severity. Subsequently conventional PCR targeting the triose phosphate isomerase gene identified 12 infections belonging to assemblage A, 86 to assemblage B and 4 infections with both assemblages. Intensity of infection and gastro-intestinal complaints were equally distributed among assemblages A and B.

INTRODUCTION

Giardia lamblia is an enteric pathogen found in a wide range of mammalian hosts, including humans (Thompson, 2000). Infection with *G. lamblia* is very common throughout the world and is considered as one of the main non-viral causes of enteric illness of man. Symptoms of *G. lamblia* infections are highly variable, and can range from asymptomatic to the presence of severe gastro-intestinal complaints (Whitty et al., 2000; De Wit et al., 2001a; Hörman et al., 2004; Amar et al., 2007). The reasons for the clinical heterogeneity of *G. lamblia* infections are not fully understood and are still subject for investigation. Presence and severity of symptoms might partly be attributed to the hosts' immune response, variation in the virulence of *G. lamblia* isolates, or a combination of both (Farthing, 1997; Thompson & Monis, 2004).

Molecular analysis have shown distinctive genetic differences in *G. lamblia* isolates from humans that can be clustered into genetic assemblages A and B (Monis et al., 2003). Previous studies have suggested an epidemiological role of the assemblages for the presence and the severity of *G. lamblia* infections (Homan & Mank, 2001; Read et al., 2002; Haque et al., 2005; Gelanew et al., 2007; Sahagun et al., 2008; Kohli et al., 2008; Pérez Córdón et al., 2008). So far, the role of the assemblages remains largely unresolved as these studies have been inconsistent with their results on associating the assemblages with the severity of disease.

In general, *G. lamblia* infections are diagnosed by microscopy and/or antigen detection in faecal samples. These methods have also been selected in previous mentioned epidemiological studies for the initial recognition of the study subjects. Real-time PCR on DNA isolated from stool samples has the potential to detect a significant number of *G. lamblia* infections, that would have remained undetected by microscopy and/or antigen detection (Ten Hove et al., 2007, 2009). Moreover, the intensity of the *G. lamblia* infections can be reflected by the numeric outcome of real-time PCR defined by Cycle threshold (Ct)-values indicate.

The present study describes the molecular epidemiology of *G. lamblia* infections detected by highly sensitive real-time PCR in returned travellers for which gastro-intestinal complaints were actively recorded.

MATERIAL AND METHODS

SAMPLE COLLECTION AND IDENTIFICATION OF GIARDIA LAMBLIA

In a prospective study on the molecular diagnostics of intestinal parasites in returned travellers, faecal samples have been collected between April 2005 and May 2006

from 2591 persons attending the travel clinic at the Institute for Tropical Medicine (ITM) in Antwerpen, Belgium. Details of the study participants about their age, gender, recent travel and gastro-intestinal complaints (diarrhoea, abdominal pain, flatulence, nausea, duration of complaints) have actively been recorded by attending physicians. A complete description of the study subjects has been presented previously (Ten Hove et al., 2009). For this study, 132 PCR positive cases were selected for epidemiological analysis after samples ($n = 17$) were excluded if mixed infections with other pathogenic intestinal parasites were detected ($n = 10$), presence of bacterial infections ($n = 7$) and/or when clinical data were missing ($n = 1$). Stool examination for the presence of *G. lamblia* was performed according to the standard routine procedures at the diagnostic laboratory of the ITM, which included microscopic examination for presence of cysts and Copro-antigen ELISAs for detection of *Giardia* (ProSpecT, Remel, Lenexa, Kansas, USA). Real-time PCR analysis for *G. lamblia* was performed at the department of Parasitology at Leiden University Medical Centre (LUMC) on DNA isolated from faeces. The Ct-value, reflecting the amount of parasitic DNA, was determined on all samples. Undetected Ct-values were redefined as 50.

OLIGONUCLEOTIDE PRIMERS ON TRIOSE PHOSPHATE ISOMERASE (TPI)

GENE

Samples in which *G. lamblia* specific DNA was detected with real-time PCR were subjects for further assemblage determination. A PCR was used for specific amplification of a triose phosphate isomerase (TPI) gene fragment. Primers were designed with Primer Express software (Applied Biosystems, Foster City, CA, USA) on the basis of TPI DNA sequences specific for assemblage A (GenBank accession no. U57897) and B (GenBank accession no. L02116). Forward primer Gi-A-1141F (5'-GCG CAC AGC ATA TCC GTA TC-3') and reverse primer Gi-A-1227R (5'-AGC CGT CAA TAT TCG GAC AC-3') amplified a 86-bp fragment of assemblage A. Forward primer GiB-940F (5'-ATG AAC GCA AGG CCA ATA AC-3') and reverse primer Gi-B-1067R (5'-GAT AGA CCA CAC CGG CTC AT-3') amplified a 127-bp fragment of assemblage B.

PCR AMPLIFICATION OF ASSEMBLAGES A AND B

Amplification reactions with the two primer pairs (Biolegio, Nijmegen, The Netherlands) were performed as a single PCR in a 25 μ L volume containing PCR buffer (HotstarTaq master mix; QIAGEN), 2.5 μ g Bovine Serum Albumin (Roche Diagnostics Nederland B.V., Almere, The Netherlands), 6.25 pmol of each forward and reverse primer, and 5.0 μ L of the DNA sample. DNA amplification consisted of 15 min at 95 $^{\circ}$ C, then 45 cycles of 95 $^{\circ}$ C for 20 s, 55 $^{\circ}$ C for 20 s, and 72 $^{\circ}$ C for 30 s. A last step at 72 $^{\circ}$ C for 5 min was used for final extension period. PCR products were run on 2%

agarose-TBE gels and visualized with ethidium bromide.

DATA ANALYSIS

Data was stored in a Microsoft Access database (Microsoft, Redmond, WA, USA) and exported into a SPSS file (SPSS Inc., Chicago, IL) for statistical analysis. Symptomatic *G. lamblia* infections were recorded as presence of diarrhoea, flatulence, abdominal pain and/or nausea. A symptoms score was established by adding a point with presence of each gastro-intestinal complaint and an additional point was added when duration of gastro-intestinal complaints was longer than two weeks. The symptoms score ranges from null (no complaints recorded) to five (all four complaints mentioned and present longer than 2 weeks). Simple *t* tests were used to compare mean differences of Ct-values and χ^2 analyses were used to compare categorical variables. The Spearmans's non-parametric correlation coefficient (ρ) was used to calculate concordance in intensity of infection as determined by Ct-values and the symptoms scores, respectively. A p-value of less than 0.05 was considered as statistically significant.

RESULTS

CHARACTERISATION OF *G. LAMBLIA* INFECTIONS

The gender of the 132 cases was evenly distributed among those with or without gastro-intestinal complaints. The ages of the study participants ranged from 1 to 77 years (median 32), with 13 persons less than 15 years and 4 persons older than 59 years. Gastro-intestinal complaints were mentioned by 63 persons, of which 48 had complaints for more than 2 weeks. Gastro-intestinal complaints of all persons were subdivided into six symptoms score categories as shown in table 5.1.

Real-time PCR for *G. lamblia* detection was performed on DNA extracted from all stool samples without prior purification and concentration of the cysts. Ct-values from the 132 cases ranged from 17.0 to 44.7 with a median of 29.8. In 29 of these real-time PCR positive samples, microscopy and copro-antigen detection was negative.

Table 5.1. Distribution of cases by complications score ranging from 0 (no gastro-intestinal complaints) to 5 (all four recorded gastro-intestinal symptoms present for more than 2 weeks) with proportions of *Giardia lamblia* positive cases and median Ct-value of *G. lamblia* real-time PCR of complications score categories.

S-score	Nr. of cases n (%)	Range Ct-values	Median Ct-values
0	69 (52.3)	21.5 – 44.7	31.5
1	6 (4.5)	25.4 – 38.6	31.0
2	9 (6.8)	24.7 – 36.4	31.8
3	16 (12.1)	23.7 – 40.2	29.8
4	16 (12.1)	19.1 – 34.4	27.7
5	16 (12.1)	17.0 – 37.4	27.7
Total	132 (100)	17.0 – 44.7	29.8

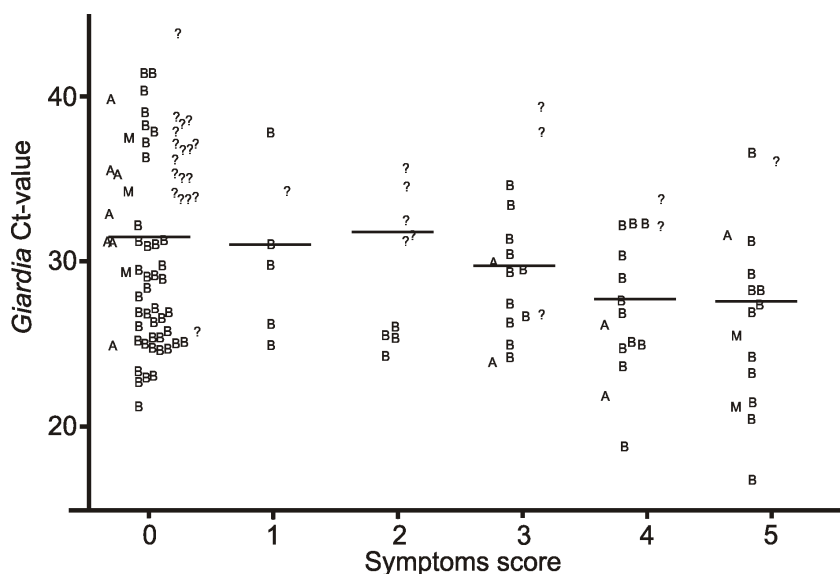
Samples from patients with symptomatic *G. lamblia* infections showed significantly lower Ct-values ($n = 63$; range: 17.0 – 40.2; median = 28.7) compared to samples from those with asymptomatic *G. lamblia* infections ($n = 69$; range: 21.5 – 44.7; median = 31.5) ($p < 0.005$). Furthermore, the increasing symptoms score corresponded with decreasing *G. lamblia* Ct-values ($n = 132$; $\rho = -0.27$; $p < 0.005$) (table 5.1).

CHARACTERISATION OF GIARDIA LAMBLIA ASSEMBLAGES

Amplification products of the TPI gene fragment were revealed in 102 of the 132 cases: 12 cases with genotypes belonging to assemblage A, 86 cases with genotypes belonging to assemblage B and in 4 cases both assemblages were identified. In 13 cases assemblages were identified with PCR while microscopy and copro-antigen detection remained negative.

Distribution of *G. lamblia* assemblages A and B infections showed no predisposition towards gender, age or regions where travellers were possibly exposed. No statistical significant relationship was determined between the assemblages and presence of gastro-intestinal symptoms or with the symptoms score. Ct-values from samples of assemblage A ($n = 12$; range: 21.7 – 39.2; median = 30.8) were similar to Ct-values from samples of assemblage B ($n = 86$; range: 17.0 – 42.2; median = 27.5) ($p = 0.40$). Figure 5.1 shows the distribution of the assemblage categories against the Ct-value and symptoms score.

Figure 5.1. Ct-values of *Giardia lamblia* positive samples (n = 132) clustered by the symptoms score with median Ct-value indicated by horizontal lines. The assemblages A, B, A/B and unknown are represented by 'A', 'B', 'M' and '?', retrospectively.



DISCUSSION

In this study, the semi-quantitative *G. lamblia* detection, reflected by Ct-values, showed significant correlation with the number of gastro-intestinal complaints of the person. The Ct-values can therefore indicate the severity of an infection. The amplification of the TPI region for assemblage detection showed feasible on samples with Ct-values around 33 and lower. With the molecular diagnostic approach, assemblages could be detected in 13 (10%) additional cases which would not have been detected if samples were selected by microscopy and antigen tests. In general assemblage B showed higher prevalence among the returned travellers. Nevertheless, no predisposition was observed of assemblages A or B towards the presence or number of gastro-intestinal symptoms. Ct-values were comparable in both assemblage groups, indicating similar parasite loads. No trend was observed for a higher presence of an assemblage in any part of the world.

With 29 (22%) additional detected *G. lamblia* infections, real-time PCR showed highly sensitive as compared to microscopy and copro-antigen tests. Subsequent differentiation of *G. lamblia* isolates can be determined by the sequence analysis of various genes, e.g. the small-subunit (SSU) rDNA, glutamate dehydrogenase (GDH),

triose phosphate isomerase (TPI) and β -giardin genes (Monis & Thompson, 2003). PCR assays amplifying TPI gene fragments proved a reliable method to distinguish assemblages A and B in human stool isolates compared to other genotype assays (Bertrand et al., 2005). The TPI PCR assay, however, could not identify assemblages in 23% of all the *G. lamblia* positive cases, in particular those with high Ct-values / low parasite shedding. Interpretations and conclusions based on the *G. lamblia* assemblages detected, should be taken with some caution until methods can be applied that will detect *G. lamblia* genotypes with a comparable sensitivity as real-time PCR. Nevertheless, if all unknown assemblages would have been replaced either by assemblage A or B, associations with age, travel destiny or symptoms would still not be observed.

Previous studies suggested that *G. lamblia* assemblages are important in the clinical outcome in children, whereas the symptoms in adults are more related to underlying conditions such as the degree of host adaptation, nutritional and immunological status (Sahagun et al., 2008). Based on the TPI gene target, Sahagun et al. showed an association for *G. lamblia* assemblage A with gastroenteritis in children of less than 5 years of age (Sahagun et al., 2008). These results concur with studies from Read et al. and Haque et al., who found the same correlation in children after assemblage typing analysis of the SSU-rDNA (Read et al., 2002; Haque et al., 2005). However, with those of 5 years and older, no association between assemblage and presence of symptoms was shown in the study from Sahagun et al, whereas Homan and Mank hypothesized the presence of more severe diarrhoea with *G. lamblia* of assemblage B after analysing a similar age group with the GDH as target gene (Homan & Mank, 2001). Study participants in this study were predominantly middle aged but as they attended a travel clinic their backgrounds were most likely very different such as immigrant, businessman, working for a NGO, etc. Background and underlying health conditions were not included in this study but are likely more important factors in adults for the differences in symptomatology, compared to possible virulent factors in *G. lamblia* genotypes.

ETHICAL APPROVAL

This study was part of a larger investigation on the diagnostic value of multiplex real-time PCR in clinical laboratory, for which approval was obtained from the ethical committees of the Institute of Tropical Medicine in Antwerp, Belgium.

CHAPTER 6

REAL-TIME PCR FOR DETECTION OF *ISOSPORA BELLII* IN STOOL SAMPLES.

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ABSTRACT

A real-time polymerase chain reaction (PCR) assay targeting the internal transcribed spacer 2 region of the ribosomal RNA gene was developed for the detection of *Isoospora belli* DNA in fecal samples, including an internal control to detect inhibition during the amplification process. The assay was performed on species-specific DNA controls (n = 27) and a range of positive (n = 21) and negative (n = 120) stool samples, and achieved 100% specificity and sensitivity. The simple fecal sample collection procedure, the high-throughput potential, and the possibility of quantification makes the *I. belli* real-time PCR assay a powerful diagnostic tool for epidemiologic studies with possibilities for extension to other helminthes and protozoa using additional molecular targets. In addition, this *Isoospora* PCR could augment the clinical laboratory diagnosis of isosporiasis, in particular, in patients with a travel history to developing countries.

INTRODUCTION

Infection with the intestinal protozoa *Isospora belli* is associated with chronic and severe diarrhea, in particular, for persons living with AIDS and other immunocompromised individuals (Ferreira, 2000; Lewthwaite et al., 2005; Atambay et al., 2007). Infections are also seen in children and travelers to tropical regions (Okhuysen, 2001; Goodgame, 2003; Jongwutiwes et al., 2007). Although symptoms are self-limiting in immunocompetent individuals, early diagnosis and treatment can shorten the period of intestinal symptoms substantially. Diagnosis is usually made 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. To improve sensitivity, we have to perform additional microscopic, concentration, and/or staining methods (Franzen et al., 1996; Lainson & da Silva, 1999; Bialek et al., 2002).

A nested polymerase chain reaction (PCR) method with Southern blot hybridization was described by Muller et al. as a helpful technique for the detection of very mild *I. belli* infections (Müller et al., 2000). However, these are very laborious procedures and, therefore, not efficient for analyzing large number of samples. Recent studies have demonstrated the detection of parasite DNA in feces with real-time PCR to be a sensitive and specific alternative technique for the diagnosis of intestinal parasitic infections (Verweij et al., 2007c; Ten Hove et al., 2007). Real-time PCR is a closed system that can provide quantitative information for up to 3 targets, including an internal control in 1 multiplex assay.

In the present study, a real-time PCR was developed for the specific detection of *I. belli* DNA in fecal samples. In addition, an internal control for the detection of possible inhibition of the amplification by fecal contaminants was included in the assay. The performance of the PCR was evaluated using a large range of DNA controls.

MATERIALS AND METHODS

CONTROLS AND SAMPLES

To establish the real-time PCR assay, we used *I. belli* control DNA extracted from an unpreserved human fecal sample in which the presence of *I. belli* oocysts was confirmed by microscopy.

Specificity of the PCR assay was evaluated using 1) DNA isolated from individual adult worms of *Schistosoma mansoni* and *Necator americanus* and also DNA of *Strongyloides stercoralis* 1st-stage larvae and *Ancylostoma duodenale* 3rd-stage

larvae, *Cyclospora cayetanensis*, *Entamoeba histolytica*, *Entamoeba dispar*, *Giardia lamblia*, *Cryptosporidium parvum*, *Enterocytozoon bieneusi*, *Encephalitozoon intestinalis* (Verweij et al., 2007c), *Dientamoeba fragilis* DNA isolated from culture (ATCC 30948), and *Toxoplasma gondii* DNA isolated from RH reference strain of *T. gondii* maintained in female Swiss Webster mice by intraperitoneal passage; 2) DNA obtained from different bacterial/yeast cultures of *Enterococcus faecalis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella enteritidis*, *Salmonella typhimurium*, *Enterobacter aerogenes*, *Yersinia enterocolitica*, *Bacillus cereus*, *Proteus mirabilis*, *Candida albicans*, *Shigella flexneri*, *Shigella dysenteriae*, *Shigella sonnei*, and *Escherichia coli* O157; and 3) 80 DNA extracts from unpreserved stool samples from different patients in which *E. histolytica* (n = 20), *E. dispar* (n = 20), *G. lamblia* (n = 20), or *C. parvum* / *Cryptosporidium hominis* (n = 20) were detected by microscopy and confirmed by specific PCR, respectively (Verweij et al., 2003a, 2004a). The specificity of the PCR assay was also tested on DNA extracts from 40 unpreserved stool samples with a negative result in microscopy using formalin–ether sediments and modified acid-fast staining, and a *Giardia* antigen test. For these negative samples, 2 subsequent stool samples from these patients tested negative by microscopy and *Giardia* antigen detection.

The PCR was further evaluated using DNA extracts of 21 stool samples from individual patients in which the microscopic examination of modified acid-fast stained fecal smears revealed *I. belli* oocysts. Eighteen stool samples originated from the Queen Elizabeth Central Hospital in Blantyre, Malawi, where HIV/AIDS is highly endemic. Three stool samples were used from travelers to Nigeria, India, and Nepal, diagnosed at the Institute of Tropical Medicine in Antwerp, Belgium (n = 2), and the Albert Sweitzer Hospital in Dordrecht, The Netherlands, respectively.

Furthermore, DNA was isolated from serial fecal dilution series with 100 oocysts to estimate the sensitivity of the PCR.

DNA ISOLATION

For DNA isolation, approximately 100 mg of unpreserved feces were suspended into 200 µL of phosphate-buffered saline containing 2% polyvinylpyrrolidone (Sigma, Steinheim, Germany). After heating for 10 min at 100 °C, suspensions were treated with sodium dodecyl sulfate–proteinase K for 2 h at 55 °C. DNA was then isolated using QIAamp Tissue Kit spin columns (QIAGEN, Hilden, Germany) (Verweij et al., 2001). If formalin-fixed fecal samples are used, the formalin has to be removed by washing/centrifuging steps with saline direct or after formalin–ether concentration. However, sensitivity of amplification is known to decrease with time of fixation (Ramos et al., 1999). Within the isolation lysis buffer, 103 plaque-forming units (PFU)/mL phocin herpes virus 1 (PhHV-1) was added to serve as an internal control (Niesters, 2002).

PCR AMPLIFICATION AND DETECTION

I. belli-specific primers and detection probe were designed using Primer Express software (Applied Biosystems, Foster City, CA) on the internal transcribed spacer 2 ribosomal RNA gene sequence (GenBank accession no. AF443614). For detection of *I. belli*, primers Ib-40F (5'-ATA TTC CCT GCA GCA TGT CTG TTT-3') and Ib-129R (5'-CCA CAC GCG TAT TCC AGA GA-3') were selected to amplify a fragment of 89 bp, which was detected by the double-labeled probe Ib-81Taq (FAM-5'-CAA GTT CTG CTC ACG CGC TTC TGG-3'-BHC1) (Biolegio, Malden, The Netherlands). For simultaneous detection of internal control, PhHV-1-specific primers and probe set consisted of forward primer PhHV-267s, reverse primer PhHV-337as, and the specific Cy5-BHQ2-labeled probe PhHV-305tq (Biolegio) (Niesters, 2002). National Center for Biotechnology Information (NCBI) BLAST search was used to test the theoretical specificity of the primers and probe.

A 10-fold dilution series of *I. belli* control DNA was used to optimize the real-time PCR and tested with and without the presence of internal control DNA to estimate the latter's influence.

For DNA amplification, 5 µL of DNA extracted from stool specimens was used as a template in a final volume of 25 µL with 2× PCR buffer (HotstarTaq master mix, QIAGEN), 5 mmol/L MgCl₂, 2.5 µg bovine serum albumin (Roche Diagnostics Nederland, Almere, The Netherlands), 60 nmol/L of each *I. belli*-specific primer, 150 nmol/L of each PhHV-1-specific primer, and 100 nmol/L of *I. belli*- and PhHV-1-specific double-labeled probes. Amplification consisted of 15 min at 95 °C followed by 50 cycles of 15 s at 95 °C and 1 min at 60 °C. Amplification, detection, and data analysis were performed with the Applied Biosystems 7500 Real-Time PCR system and Sequence Detection Software version 1.2.2.

RESULTS

In the NCBI BLAST search, primers and probe showed 100% specificity for *I. belli*.

The real-time PCR was optimized using a 10-fold dilution series of *I. belli*-positive control DNA. The cycle threshold (Ct) values obtained from testing the dilution series of *I. belli* DNA in both the individual *I. belli* assay and the multiplex assay with the internal control showed no systemic deviation in amplification curves, and the same analytical sensitivity was achieved. The individual performance of the assays was not influenced by the presence of DNA from the internal control.

The specificity of the real-time multiplex PCR was evaluated using a range of parasite and bacterial control DNAs (n = 27), 80 DNA extracts derived from feces positive for *E. histolytica*, *E. dispar*, *G. lamblia*, or *C. parvum*/*C. hominis*, and 40 DNA extracts

derived from feces of individuals with no known history of parasitic infections. No amplification of *I. belli*-specific DNA was detected in any of these 147 samples; only the amplification of the internal control was detected at the expected threshold cycle of approximately 30.

I. belli-specific amplification was detected in all 21 samples in which *I. belli* was detected by microscopic examination, with Ct values of between 22.8 and 30.5 with a median threshold of 25.8 cycles. One *I. belli* oocyst was estimated as the detection limit of the isolation and PCR procedure. Fecal suspensions containing 100-, 10-, or 1 oocyst corresponded with Ct values 30.0, 33.2, and 36.3, respectively.

DISCUSSION

Among immunocompromised individuals, especially those living with AIDS, the most important opportunistic intestinal parasites include *Cryptosporidium* spp., microsporidia, and *I. belli*. Additional staining techniques are required for microscopic detection of all these organisms. Nevertheless, light infections can still be easily missed. Already, highly sensitive and specific real-time PCR procedures have been validated for the detection of *Cryptosporidium* spp. (Verweij et al., 2004a; Ten Hove et al., 2007), *E. bienersi*, and *Encephalitozoon* spp. (Verweij et al., 2007c).

In the present study, an *I. belli* real-time PCR was developed to complete the series of molecular diagnostic assays for the detection of opportunistic intestinal parasites. Using a large range of control DNA and stool samples, the real-time PCR achieved 100% specificity. In a selected group of 21 fecal samples from patients in which *I. belli* was shown by microscopy, the real-time PCR showed a sensitivity of 100%. Considering the low Ct values obtained in these microscopy-positive samples and the detection limit of 1 oocyst in a fecal suspension, this real-time PCR has the potential to detect *I. belli* infections in patients shedding very low numbers of oocysts. A prospective study on the sensitivity of the real-time PCR as compared by microscopic techniques is planned for the near future.

In Western countries, isosporiasis is diagnosed occasionally, and those with higher risk are travelers and immigrants. Nevertheless, the true number of cases could be underestimated, and more cases are probably detected in routine screening of specific patient groups with real-time PCR for *I. belli*. This real-time PCR could be part of routine posttravel screening multiplex PCR extended with other targets such as *C. cayetanensis* (Verweij et al., 2003c).

Obviously, this real-time PCR will not be appropriate as a routine diagnostic tool in clinical settings in endemic areas where resources of the laboratories are often limited. However, the high-throughput potential and species-specific quantification as a measure of intensity of infection can be used, together with other collected

health and demographic indicators, as a powerful tool for epidemiologic studies or to monitor effectiveness of treatment in *I. belli* infections (Pape et al., 1989; Verdier et al., 2000). Stool samples mixed with ethanol allow the samples to be stored and transported at room temperature to laboratories with the appropriate facilities for DNA extraction and detection. Moreover, once DNA is extracted, the real-time PCR assay can be extended to other helminths or protozoa using additional molecular targets. In addition, up to 4 molecular targets with different fluorescent labels can be combined as a multiplex real-time PCR assay, leading to considerable reduction in reagent costs.

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CHAPTER 7

MULTIPLEX DETECTION OF *ENTEROCYTOZOOM BIENEUSI* AND
ENCEPHALITOZOOM SPP. IN FECAL SAMPLES USING REAL-TIME PCR.

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ABSTRACT

A multiplex real-time polymerase chain reaction (PCR) method was developed for the simultaneous detection of *Enterocytozoon bieneusi* (n = 30) and *Encephalitozoon* spp. (n = 3) in stool samples. The multiplex PCR also included an internal control to detect inhibition of the amplification by fecal constituents in the sample. The assay was performed on species-specific DNA controls (n = 22) and a range of well-defined stool samples (n = 140), and it achieved 100% specificity and sensitivity. The use of this assay in a diagnostic laboratory offers the possibility of introducing DNA detection as a feasible technique in the routine diagnosis of intestinal microsporidian infections.

INTRODUCTION

Microsporidia are small obligate intracellular parasites originally considered to be primitive eukaryotic protozoa but are recently reclassified with the fungi. Of the more than 1000 species of microsporidia, 14 species are known to infect humans of which *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis* are responsible for most infections (Didier, 2005). *E. bieneusi* and *E. intestinalis* infections have been frequently recognized as a cause of severe diarrhea in persons with AIDS. Recently, intestinal microsporidiosis is increasingly diagnosed in other immunocompromised individuals such as organ transplant recipients, as well as in children, travelers, and elderly (Gomez Morales et al., 1995; Wanke et al., 1996; Raynaud et al., 1998; Gainzarain et al., 1998; Lopez-Velez et al., 1999; Müller et al., 2001; Lores et al., 2002; Mungthin et al., 2005; Leelayoova et al., 2005; Wichro et al., 2005). Microscopic detection of the very small spores of microsporidia species in stool samples requires additional staining techniques, usually optical white stain (Van Gool et al., 1994) or modified trichrome stain (Garcia, 2002). However, detection and differentiation of the spores from other fecal components after nonspecific optical white staining can still be difficult. Although the modified trichrome stain is more specific, interpretation of the 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 determination, which is important, because treatment with albendazole is effective for *E. intestinalis* infection but not for *E. bieneusi* infections (Didier, 2005). An alternative drug, fumagillin, which is effective for both species (Didier, 2005), is also known to be very toxic and therefore not considered appropriate for the treatment of *E. intestinalis* infections.

Although polymerase chain reaction (PCR)-based methods have been successfully used for the detection of microsporidian infections (Katzwinkel-Wladarsch et al., 1996; Kock et al., 1997; Franzen & Müller, 1999; Notermans et al., 2005), their application in routine diagnosis is still very limited. Introduction of PCR-based methods has been hindered by difficulties in the DNA extraction from fecal samples. Moreover, the amplification and detection of DNA was prone to contamination as well as time consuming and expensive. In recent years, however, the isolation of parasitic DNA from fecal samples has been improved and simplified (Verweij et al., 2001; Subrungruang et al., 2004). The introduction of real-time PCR using fluorescent detection probes (Klein, 2002) can reduce the risk of contamination, labor time, and reagent costs through the possibility of combining assays for the detection of different targets into one assay. Recently, real-time methods for the detection of *Encephalitozoon* spp. and *E. bieneusi* were published (Wolk et al., 2002; Menotti et al., 2003b, 2003a). However, because they were all separate assays without an

internal control, the advantages of using real-time PCR were not fully exploited.

Therefore, a multiplex real-time PCR was developed for the simultaneous detection of *E. bieneusi* and *Encephalitozoon* spp. in fecal samples. In addition, an internal control for the detection of possible inhibition of the amplification by fecal contaminants was included in the assay. The performance of the assay was evaluated using a range of control samples.

MATERIAL AND METHODS

CONTROLS AND SAMPLES

E. bieneusi control DNA was obtained from a human stool sample in which *E. bieneusi* was detected by fluorescence microscopy after optical white staining and confirmed by electron microscopy, and *E. intestinalis* control DNA was obtained from a cell culture of *E. intestinalis* originally isolated from human feces.

Thirty-three stool samples were selected in which microsporidian spores were detected by fluorescence microscopy after optical white staining, and *E. bieneusi* (n = 30) and *Encephalitozoon* spp. (n = 3) were confirmed by conventional PCR, which has been used routinely in our laboratory since 1996 (Katzwinkel-Wladarsch et al., 1996). All samples used were from different patients and collected from 1996 onward and stored at -20 °C (table 7.1). Also, 60 unpreserved stool samples were tested from patients with a negative result in microscopy of formalin–ether sediments, modified acid-fast staining, and *Giardia*-antigen test. In these negative samples, 2 subsequent stool samples from these patients tested negative by all conventional methods.

Table 7.1. *E. bienewsi* and *Encephalitozoon* spp. real-time PCR results in fecal samples from different patient groups.

Underlying condition	No. (n)	Positive PCR result	Ct value
HIV infection	16	<i>E. bienewsi</i>	15.8–36.5
Kidney transplantation	6	<i>E. bienewsi</i>	18.9–24.8
Hypogammaglobulinemia	1	<i>E. bienewsi</i>	21.6
Adoption (1 and 4 years old)	2	<i>E. bienewsi</i>	29.8; 34.8
Traveler	1	<i>E. bienewsi</i>	23.1
Unknown	4	<i>E. bienewsi</i>	17.9–21.6
HIV infection	2	<i>Encephalitozoon</i> spp.	22.4; 24.7
Unknown	1	<i>Encephalitozoon</i> spp.	34

Serial 10-fold dilution series of DNA extracted from each pathogen were tested with and without the presence of internal control DNA to estimate the influence of the internal control. Each dilution series was also tested with and without the other target to assess the ability to detect mixed infections.

Furthermore, DNA isolated from a serial dilution series of 100 000 *E. bienewsi* spores into negative feces was used to estimate the sensitivity of the PCR.

The specificity of the PCR was tested using *Entamoeba histolytica*, *Entamoeba dispar*, *Giardia lamblia*, *Cryptosporidium parvum*, or *Cyclospora cayetanensis* DNA as template. *E. histolytica* DNA was obtained from an axenic culture of *E. histolytica* HM1 strain, and *E. dispar* DNA from a polyxenic culture from human feces. *G. lamblia* DNA was isolated from purified *G. lamblia* cysts, and *C. parvum* DNA from purified *C. parvum* oocysts (Waterborne, New Orleans, LA). *C. cayetanensis* DNA was isolated from a human stool sample known to contain *C. cayetanensis* oocysts and confirmed with *C. cayetanensis*-specific real-time PCR (Verweij et al., 2003c). Specificity of the assay was also tested on DNA obtained from 17 different bacterial/yeast cultures: *Bacillus cereus*, *Campylobacter jejuni*, *Campylobacter upsaliensis*, *Candida albicans*, *Escherichia coli* O157, *Enterobacter aerogenes*, *Enterococcus faecalis*, *Proteus mirabilis*, *Pseudomonas aerogenosa*, *Salmonella enteritidis*, *Salmonella typhimurium*, *Shigella boydii*, *Shigella dysenteriae*, *Shigella flexneri*, *Shigella sonnei*, *Staphylococcus aureus*, and *Yersinia enterocolitica*. All isolates were grown and typed biochemically using routine bacteriologic procedures.

Furthermore, 80 stool samples from different patients were tested in which *E. histolytica* (n = 20), *E. dispar* (n = 20), *G. lamblia* (n = 20), or *C. parvum* / *C. hominis* (n = 20) was detected by microscopy and confirmed by specific PCR, respectively

(Verweij et al., 2003a, 2004a).

MICROSCOPY

Microscopic examination for the presence of ova and cysts was performed routinely by examination of iodine stained wet mount after formal-ether concentration using magnification $\times 400$. Modified acid-fast staining was performed on direct smears and formal-ether concentrates for detection of coccidian parasites. Fluorescence microscopy after optical white staining was performed on direct smears and saline-ether concentrates for the detection of microsporidian spores (Van Gool et al., 1994).

DNA ISOLATION

For DNA isolation, 200 μL of feces suspension (≈ 1 g/mL of phosphate-buffered saline containing 2% polyvinylpolypyrrolidone, Sigma-Aldrich, Zwijndrecht, The Netherlands) was heated for 10 min at 100°C . After sodium dodecyl sulfate-proteinase K treatment (2 h at 55°C), DNA was isolated with the QIAamp Tissue Kit spin columns (QIAGEN, Hilden, Germany) (Verweij et al., 2001). If formalin-fixed fecal samples are used, the formalin has to be removed by washing/centrifuging steps with saline direct or after formalin-ether concentration. However, sensitivity of amplification is known to decrease with time of fixation.

In each sample, 103 PFU/mL phocine herpes virus 1 (PhHV-1) was added within the isolation lysis buffer to serve as an internal extraction and amplification control (Niesters, 2002).

PCR AMPLIFICATION AND DETECTION

E. bieneusi-specific PCR primers and a detection probe were chosen using Primer Express software (Applied Biosystems, Foster City, CA) on the internal transcribed spacer (ITS) sequence for *E. bieneusi* (GenBank accession no. AF101198) such that a 103-bp fragment within the ITS sequence should be amplified and detected for *E. bieneusi* specifically. The *E. bieneusi*-specific primers and probe set consisted of forward primer EbITS-89F, reverse primer EbITS-191R, and *E. bieneusi*-specific double-labeled probe EbITS-114revT (Eurogentec, Belgium).

Encephalitozoon spp. PCR primers and detection probe were designed using Primer Express software (Applied Biosystems) on the small subunit ribosomal RNA gene sequence for *E. intestinalis* (GenBank accession no. U09929) such that *Encephalitozoon* spp. DNA would be specifically amplified and detected. The forward primer MSP-F and reverse primer Eint213R amplified a 214-bp fragment inside the SSU rRNA gene. The *Encephalitozoon* spp.-specific double-labeled probe Eint82rev (Eurogentec) was used to detect *Encephalitozoon* spp.-specific amplification.

PhHV-1 specific primers and probe (Niesters, 2002) set consisted of forward primer

PhHV-267s, reverse primer PhHV-337as, and specific double-labeled probe PhHV-305tq (Biolegio, The Netherlands).

Amplification reactions were performed in a volume of 25 µL with PCR buffer (HotstarTaq master mix, QIAGEN), 5 mmol/L MgCl₂, 2 pmol of each *E. bienersi*-specific primer, 6.25 pmol of each *E. intestinalis*-specific primer, 3.75 pmol of each PhHV-1-specific primer, 2.5 pmol of *E. bienersi*-specific double-labeled probe, 2.5 pmol of *Encephalitozoon* spp.-specific double-labeled probe, 2.5 pmol of PhHV-1-specific double-labeled probe, and 5 µL of the DNA sample. Amplification consisted of 15 min at 95 °C followed by 50 cycles of 15 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. Amplification, detection, and data analysis were performed with the I-cycler real-time detection system (BioRad, Hercules, CA). Fluorescence of FAM, Yakima Yellow, and CY5 was measured at their respective wavelengths during the annealing step of each cycle. All primers and detection probes are described in table 7.2.

Table 7.2. Oligonucleotide primers and detection probes for real-time PCR for the simultaneous detection of *E. bienersi* and *Encephalitozoon* spp. and PhHV-1 as an internal control.

Target organism	Oligonucleotide name	Oligonucleotide sequence	Genbank accession no. or literature reference of target sequence
<i>E. bienersi</i>	EbITS-89F	TGTGTAGGCGTGAGAGTGATCTG	AF101198
	EbITS-191R	CATCCAACCATCACGTACCAATC	AF101198
	EbITS-114revT	FAM-5'-CACTGCACCCACATCCCTCACCCCTT-3'-eclipse	U09929
<i>Encephalitozoon</i> spp.	Eint227R	CTAGTTAGGCCATTACCCTAACTACCA	U09929
	Eint227R	CTAGTTAGGCCATTACCCTAACTACCA	U09929
	Eint82Trev	Yakima Yellow-5'-CTATCACTGAGCCGTCC-3'-eclipse	U09929
Phocine herpes virus 1	PhHV-267s	5'-GGGCGAATCACAGATTGAATC-3'	Niesters, 2002
	PhHV-337as	5'-GCGGTTCCAAACGTACCAA-3'	Niesters, 2002
	PhHV-305tq	Cy5 5'-TTTTATGTGTCCGCCACCATCTGGATC-3'-BHQ2	Niesters, 2002

RESULTS

The specificity of the real-time multiplex PCR was evaluated using a range of controls: DNA from *E. histolytica*, *E. dispar*, *G. lamblia*, *C. parvum*, or *C. cayetanensis*, and from *B. cereus*, *C. jejuni*, *C. upsaliensis*, *C. albicans*, *E. coli* O157, *E. aerogenes*, *E. faecalis*, *P. mirabilis*, *P. aerogenosa*, *S. enteritidis*, *S. typhimurium*, *S. boydii*,

S. dysenteriae, *S. flexneri*, *S. sonnei*, *S. aureus*, and *Y. enterocolitica*. In none of these samples amplification was detected with either of the 2 assays. Furthermore, 80 DNA extracts derived from feces positive for *E. histolytica* (n = 20), *E. dispar* (n = 20), *G. lamblia* (n = 20), or *C. parvum/C. hominis* (n = 20), and 60 DNA extracts derived from feces from individuals with no known history of parasitic infections were tested. No amplification was detected of *E. bieneusi* or *Encephalitozoon* spp. DNA in any of these samples; only the amplification of the internal control was detected at the expected threshold cycle of approximately 33.

The cycle threshold (Ct) values obtained from testing the dilution series of each pathogen in the individual assay and the multiplex assay were similar, and the same analytical sensitivity was achieved. Thus, individual performance of the assays was not influenced by the presence of DNA from the internal control or by the presence of DNA from the other targets.

One *E. bieneusi* spore was estimated as the detection limit of the isolation and PCR procedure.

E. bieneusi-specific amplification was detected in 30 of 33 DNA samples isolated from feces known to contain microsporidian spores. Threshold cycles (Ct values) were found between 15.8 and 36.5 (table 7.2) with a median threshold of 21.6 cycles. *Encephalitozoon* spp.-specific amplification was detected in the other 3 samples in which microsporidian spores were detected by microscopic examination, with Ct values of 22.4, 24.7, and 34.0, respectively.

DISCUSSION

The use of labor-intensive additional staining techniques for the microscopic diagnosis of microsporidia infections will often be limited to samples of severe immune-suppressed patients. Many laboratories will not see as many of these patients because of the success of highly active anti-retroviral therapy (HAART) therapy in HIV-infected individuals. As a consequence, gaining expertise is difficult because positive findings will be scarce. Therefore, many infections in patients under relatively mild immune suppressed conditions and immunocompetent patients will probably stay undiagnosed. The multiplex real-time PCR for the detection of *E. bieneusi* and *Encephalitozoon* spp. presented in this study gives a useful alternative for the labor-intensive and expertise-dependent microscopic methods.

Using well-defined DNA and stool samples as control, the multiplex real-time assay for the detection of *E. bieneusi* and *Encephalitozoon* spp. achieved specificity of 100%. In all samples tested in which microscopy revealed the presence of microsporidian spores, specific amplification was detected for *E. bieneusi* (n = 30) or *Encephalitozoon* spp. (n = 3). There was no difference in the performance of the

amplification of the specific targets in the individual assays as compared with the multiplex PCR so the multiplex PCR could be used with equal confidence to the individual assays. PCR inhibition by fecal constituents is known to be a serious problem (Monteiro et al., 1997). However, in stool samples without a known history of parasitic infection and in stool samples with *E. histolytica*, *E. dispar*, *G. lamblia*, or *C. parvum* / *C. hominis*, only the amplification of the internal control was detected. Hence, there was no evidence of inhibition of the amplification in any of these samples using this DNA isolation method.

The *Encephalitozoon* spp. primers and probe were designed on the known sequence of *E. intestinalis*. According to NCBI BLAST, however, also *Encephalitozoon hellem* and *Encephalitozoon cuniculi* will be amplified and detected with this primer/probe combination. Although determination to the genus level will be sufficient in clinical practice, species-specific differentiation can be achieved by sequencing of the PCR product.

E. intestinalis is also known to cause extraintestinal disease such as infections of the pulmonary and urinary tract, as well as disseminated infections. Although we have not focused on the detection of microsporidium in nonintestinal samples, we expect that the described PCR can also be used on these samples. Laboratories that have this specific diagnostic question would have to use their own validated protocols for the isolation of parasitic DNA from these samples and use this DNA in the described PCR.

Detection of parasite DNA has the potential to be more sensitive as compared with microscopy; this has already been shown for *G. lamblia*, *E. histolytica*/*E. dispar*, *C. parvum* / *C. hominis*, and *E. bieneusi* using (real-time) PCR (Webster et al., 1996; Morgan et al., 1997; Ghosh et al., 2000; Menotti et al., 2003a; Verweij et al., 2003a, 2003b, 2004a). Considering the estimated detection limit of 1 spore in the PCR and the low Ct values obtained in these microscopy-positive samples, this multiplex real-time PCR for the detection of *E. bieneusi* and *Encephalitozoon* spp. certainly has the potential to detect microsporidia infections in patients shedding very low numbers of spores.

Previously, we already postulated on the tremendous impact the implementation of automated DNA isolation procedures and other multiplex assays for the detection of parasites, viruses, and bacteria that cause diarrhea will have on the differential laboratory diagnosis of diarrheal diseases (Verweij et al., 2004a). The multiplex real-time PCR described here is a sensitive and specific method for the detection of *E. bieneusi* and *Encephalitozoon* spp. and offers the possibility of introducing DNA detection as a feasible technique in the routine diagnosis of intestinal microsporidian infections and can also be used as a part of a series of other multiplex assays in the differential diagnosis of diarrhea-causing pathogens.

CHAPTER 8

CHARACTERIZATION OF GENOTYPES OF *ENTEROCYTOZOOM BIENEUSI* IN IMMUNOSUPPRESSED AND IMMUNOCOMPETENT PATIENT GROUPS

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ABSTRACT

A retrospective phylogenetic analysis was performed on isolates of *Enterocytozoon bieneusi* to characterize the genotypes in different patient cohorts. Fifty-seven isolates, collected from patients living in Malawi and The Netherlands, were classified by age and immune status of the hosts. Sequence analysis of the internal transcribed spacer (ITS) region identified 16 genotypes; nine have not previously been described. Genotypes K and D were most prevalent among patient groups, whereas genotype C was restricted to transplantation patients receiving immunosuppressives and genotype B showed a predisposition towards patients living with HIV/AIDS. Different genotypes showed more dispersion among isolates from Malawi compared with those from The Netherlands. A constructed map estimating the genealogy of the ITS region reveals a dynamic evolutionary process between the genotypes.

INTRODUCTION

The phylum Microsporidia comprises approximately 1,200 species, 14 of which are known to infect humans. Most infections are caused by *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis* (Didier, 2005). Microsporidiosis has increasingly gained interest during the AIDS epidemic after being recognized as the etiological agent causing persistent diarrhea and systemic disease (Desportes et al., 1985). Highly active antiretroviral therapy (HAART) significantly reduced the prevalence of intestinal microsporidiosis in HIV-infected persons (Conteas et al., 2000). However, in developing countries, enteric microsporidiosis still remains a major health problem due to high prevalence of HIV/AIDS in combination with underfinanced health care systems (Endeshaw et al., 2006). With heightened awareness, intestinal microsporidiosis has been increasingly diagnosed in transplant recipients, children, elderly, and travelers (Wanke et al., 1996; 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* can also be present asymptotically is accumulating (Mathis et al., 2005; Nkinin et al., 2007).

Characterization of microsporidial genotypes elucidates the dynamics of microsporidial infections in different human and animal populations. Currently, *E. bieneusi* genotypes are based on the heterogeneity of the internal transcribed spacer (ITS) of the rRNA gene (Mathis et al., 2005). Only a proportion of the described genotypes were isolated strictly from humans; various other genotypes have been isolated both from humans and animals, drawing attention to the zoonotic potential of the parasite and a possible degree of host specificity for at least some genotypes (Sulaiman et al., 2003a, 2004). Differences in the prevalence of various genotypes were also recognized between HIV-negative and HIV-positive individuals (Liguory et al., 1998).

Molecular epidemiological data of infections of *E. bieneusi* in transplant recipients, children, and immunocompetent persons remain limited. More so, previous studies on isolates of *E. bieneusi* were derived from microscopy-positive stool samples and therefore are biased towards cases with high parasite loads. Therefore, we undertook a retrospective study of isolates of *E. bieneusi* from patient groups with different clinical and demographic backgrounds using a highly sensitive and specific real-time PCR detection method (Verweij et al., 2007c). These isolates were subsequently genotyped by sequence analysis of the ITS region and analyzed for possible association with different patient groups.

MATERIAL AND METHODS

ISOLATES OF ENTEROCYTOZOOM BIENEUSI.

Fifty-seven fecal-DNA samples were selected in which *E. bieneusi* was detected using real-time PCR (Verweij et al., 2007c). Samples from Dutch patients were collected from 1996 onwards at the Leiden University Medical Center. Additionally, samples were collected between 2003 and 2004 from patients examined at the Queen Elizabeth Central Hospital (QECH) in the city of Blantyre, Malawi (table 8.1). All DNA samples were from individual patients and stored at -20°C.

DNA AMPLIFICATION.

Genotyping primers specific for *E. bieneusi* were designed using Primer Express software (Applied Biosystems, Foster City, CA, USA) on the ITS-sequence (GenBank accession no. AF101200). The forward primer Eb-80F (5'-GTT GGA GAA CCA GCT GAA GGT-3') and reverse primer Eb-375R (5'-ATA CAC CTC TTG ATG GCA CCC T-3') amplified a 296 base-pair fragment. Amplification reactions were performed in 50 µL containing 2' PCR buffer (HotstarTaq master mix, QIAgen, Venlo, The Netherlands), 5 µg Bovine Serum Albumin (Roche Diagnostics Nederland, Almere, The Netherlands), 0.5 µM of each primer (Biolegio, Malden, The Netherlands), and 5 µL of the DNA sample. DNA amplification was carried out on an I-cycler Thermal cycler (Biorad, Hercules, CA, USA) operated at 95 °C for 15 min, then 36 cycles of 94 °C for 20 s, 55 °C for 20 s, and 72 °C for 30 s. A last step at 72 °C for 5 min was used for final extension period.

Table 8.1. Demographic and clinical features (NA = not available) of male (M) and female (F) patients from The Netherlands (NL) and Blantyre, Malawi (MA) with characterized *Enterocytozoon bieneusi* genotypes.

Patient No.	Source	Age	Gender	Immune Status (additional notes)	Genotype
1	NL	30	M	HIV+	S8
2	NL	38	M	HIV+	B
3	NL	39	M	HIV+	D
4	NL	41	M	HIV+	B
5	NL	43	M	HIV+	K
6	NL	55	M	HIV+	B
7	NL	NA	NA	HIV+	B
8	NL	33	M	Kidney transplant recipient	C
9	NL	38	F	Kidney transplant recipient	C
10	NL	52	M	Kidney transplant recipient	C
11	NL	58	F	Kidney transplant recipient	C
12	NL	68	M	Kidney transplant recipient	C
13	NL	53	F	Colitis ulcerosa	S9
14	NL	43	M	Hypogammaglobulinaemia	K
15	NL	1	F	NA (adoption child)	K
16	NL	27	F	NA (traveler)	A
17	NL	33	F	NA (resident of Cuba)	K
18	NL	49	F	NA (abdominal discomfort)	S7
19	NL	50	M	NA	K
20	NL	NA	NA	NA	D
21	MA	1	M	HIV+	D
22	MA	1	F	HIV+	S5
23	MA	2	M	HIV+	K
24	MA	2	M	HIV+	D
25	MA	2	M	HIV+	S2
26	MA	2	F	HIV+	D
26	MA	2	F	HIV+	K
28	MA	3	M	HIV+	S2
29	MA	7	F	HIV+	K
30	MA	23	M	HIV+	K
31	MA	23	M	HIV+	AF502396
32	MA	24	F	HIV+	S3
33	MA	26	M	HIV+	S5
34	MA	27	F	HIV+	K
35	MA	28	F	HIV+	S5
36	MA	29	F	HIV+	S2
37	MA	30	M	HIV+	S2
38	MA	30	M	HIV+	S2
39	MA	32	F	HIV+	S5
40	MA	33	M	HIV+	K
41	MA	35	M	HIV+	K
42	MA	35	M	HIV+	S4
43	MA	35	F	HIV+	S6
44	MA	37	F	HIV+	S2
45	MA	37	F	HIV+	S2
46	MA	38	M	HIV+	S3
47	MA	38	F	HIV+	S2
48	MA	39	F	HIV+	AY371283
49	MA	39	M	HIV+	S2
50	MA	57	M	HIV+	S2
51	MA	1	M	HIV neg	S2
52	MA	1	F	HIV neg	K
53	MA	2	M	HIV neg	S1
54	MA	2	M	HIV neg	S6
55	MA	1	M	NA	K
56	MA	1	F	NA	D
57	MA	3	M	NA	S1

DNA SEQUENCING.

Ten µl of the PCR products were visualized on ethidium bromide-stained 2% agarose gels. Amplification products were purified from 40 µL of the reaction mixture with the High Pure PCR Product Purification Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. DNA concentration was measured with Nanodrop ND-1000 UV-Vis Spectrophotometer (Nanodrop Technologies, Delaware, USA). Both the forward and reverse strands were sequenced with the primers used for the PCR. Sequence analysis was performed by the Leiden Genome Technology Centre (LGTC, Leiden, The Netherlands) on an ABI 3700 DNA Analyzer (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). Sequence results were analyzed using Vector Advance 10, ContigExpress (Invitrogen, Breda, The Netherlands). To test the robustness of the sequencing method three stool specimens produced on subsequent days were obtained from patient no. 18 (table 8.1) and subjected to sequence analysis.

SEQUENCE ANALYSIS.

A BLASTN-search on NCBI database was performed with the nucleotide sequences of the isolates. Published ITS sequences representing *E. bieneusi* genotypes E, F, G, H, I, J, L, M, N, O, P, and Q, were included. All sequences were aligned using CLUSTALX 2.0.10 software (Larkin et al., 2007) with default parameters. The aligned sequences were mapped with TCS 1.2.1 software to estimate gene genealogies (Clement et al., 2000). The cladogram was made by the TREEVIEW program provided by Roderic D.M. page at the Division of Environmental and Evolutionary, Institute of Biomedical and Life Sciences, University of Glasgow.

RESULTS

The amplified PCR fragments from clinical samples were sequenced directly after purification on spin columns, without prior cloning into a vector. The robustness of this method was tested using DNA isolated from three stool specimens produced on separate days by patient no. 18. The ITS nucleotide sequences of all three isolates showed 100% similarity.

The nucleotide sequences of the ITS fragment of all 57 DNA isolates revealed 16 distinct *E. bieneusi* genotypes. The polymorphisms were fixed at 22 positions in the ITS sequence (table 8.2). Nine of the sequences were identified as new genotypes: 6 (S1 – S6) were from Malawi and three (S7 – S9) were from The Netherlands (table 8.1). These nine genotypes were related to genotypes of already published sequences of *E. bieneusi* isolated from humans and animals (figure 8.1). Genotype C was identified in all five isolates from kidney transplant recipients and was not seen in any of the other patients. The remaining six sequences were identical with

previously found genotypes A, B, D, K, and unnamed genotypes with GenBank Accession Numbers AF502396 and AY371283 (figure 8.1).

Table 8.2. Polymorphic sites in the internal transcribed spacer sequences of *E. bieneusi* isolates.

Position no.	102	112	121	133	139	146	153	157	173	177	189	190	193	201	223	235	236	237	239	251	253
K	C	A	C	G	C	C	C	G	G	A	G	C	C	C	C	A	C	C	C	C	C
S6	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.
S9	A	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AY371283 a	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.
D	.	.	.	.	.	.	A	.	.	G	.	.	.	.	.	.	.	.	.	.	.
A	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B	.	.	.	A	.	.	.	.	.	.	.	.	T	.	.	.	.	.	T	.	.
S5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.
AF502396 a	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	T	.	.
S1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T	.
S4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	T	.	.
S2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	T	T	.
S3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	T	.
C	.	.	A	.	.	T	.	A	.	.	A	.	.	.	.	.	.	.	T	.	A
S8	.	.	A	.	.	.	.	A	.	.	A	.	.	.	.	.	G	.	T	.	A
S7	.	C	.	A	G	.	.	A	A	.	A	T	T	T	.	.	.	A	T	.	.

a AY371283 and AF502396 are unnamed genotype.

Table 8.3. Numbers of isolates characterized in this study and in previous reports, sorted by country of origin, host and immune status if the host (Breitenmoser et al., 1999; Liguory et al., 2001; Tumwine et al., 2002; Sadler et al., 2002; Sulaiman et al., 2003b, 2003a, 2004; Leelayoova et al., 2005, 2006; Lobo et al., 2006; Santin et al., 2006; Sak et al., 2008) . (NA = not available)

Report	Source	Host	Immune status	Genotype															
				K	S2	S6	S1	D	S5	S3	S4	AF	AY	S9	C	S8	B	A	S7
This report	Malawi	Children	competent	1	1	1	1												
	Malawi	Children	NA	1			1	1											
	Malawi	Children	HIV pos	3	2			3	1										
	Malawi	Adults	HIV pos	4	8	1				3	2	1	1	1					
This report	Netherlands	Child	NA	1															
	Netherlands	Adults	impeded	1										1	5				
	Netherlands	Adults	HIV pos	1				1								1	3		
	Netherlands	Adults	NA	2														1	1
	Netherlands	NA	HIV pos														1		
	Netherlands	NA	NA					1											
(Tumwine et al. 2002)	Uganda	Children	NA	6								4							
(Leelayoova et al. 2005)	Thailand	Children	competent															9	
(Leelayoova et al. 2006)	Thailand	Adults	HIV pos					6											
(Sulaiman et al. 2003a)	Peru	Humans	HIV pos	18				9										35	
(Breitenmoser et al. 1999)	Switzerland	Humans	HIV pos												2		8	3	
(Sadler et al. 2002)	England	Humans	HIV pos	1				1									11		
(Liguory et al. 2001)	France	Humans	HIV pos	9													66		
	France	Humans	impeded	2											9				
	France	Humans	competent	1											1				
(Santin et al. 2006)	Colombia	Cats		2															
(Lobo et al. 2006)	Portugal	Dog						1											
(Sak et al. 2008)	Czech	Pigs						2											
(Sulaiman et al. 2003b)	USA	Wildlife						9											
(Sulaiman et al. 2004)	USA	Cattle		6															

The relatedness of the isolates, grouped by its sequence identity, are showed in the phylogenetic tree (figure8.2). The GenBank accession numbers assigned to the sequences determined in this study are as follows: genotype S1, FJ439677; genotype S2, FJ439678; genotype S3, FJ439680; genotype S4, FJ439679; genotype S5, FJ439681; genotype S6, FJ439682; genotype S7, FJ439683; genotype S8, FJ439684; genotype S9, FJ439685.

Figure 8.1. Map constructed with TCS 1.2.1 software to estimate the gene genealogies of ITS sequences of *Enterocytozoon bieneusi*. The mapped genotypes were derived from isolates in this study (grey boxes) and from the NCBI database (white boxes). Genotypes E, G, F, M, O and H have been isolated from pigs and genotypes J, I and N from cattle. Each line between ellipses and/or circles represents a single nucleotide conversion.

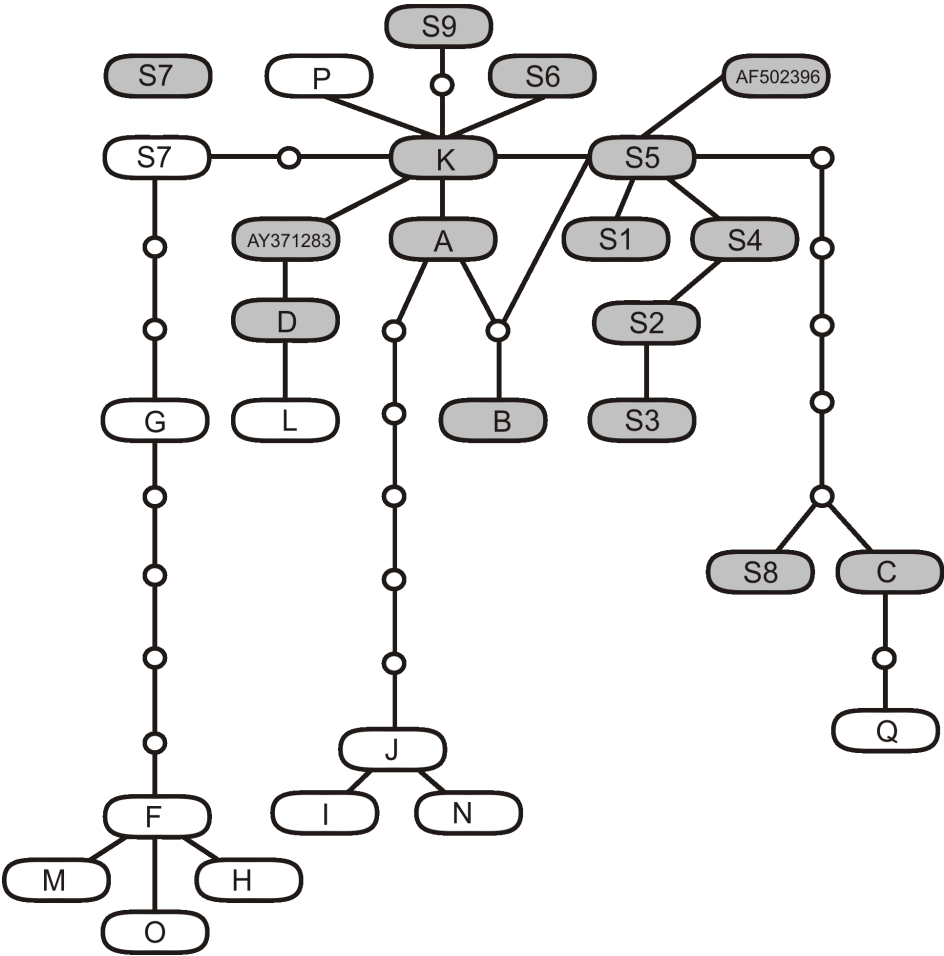
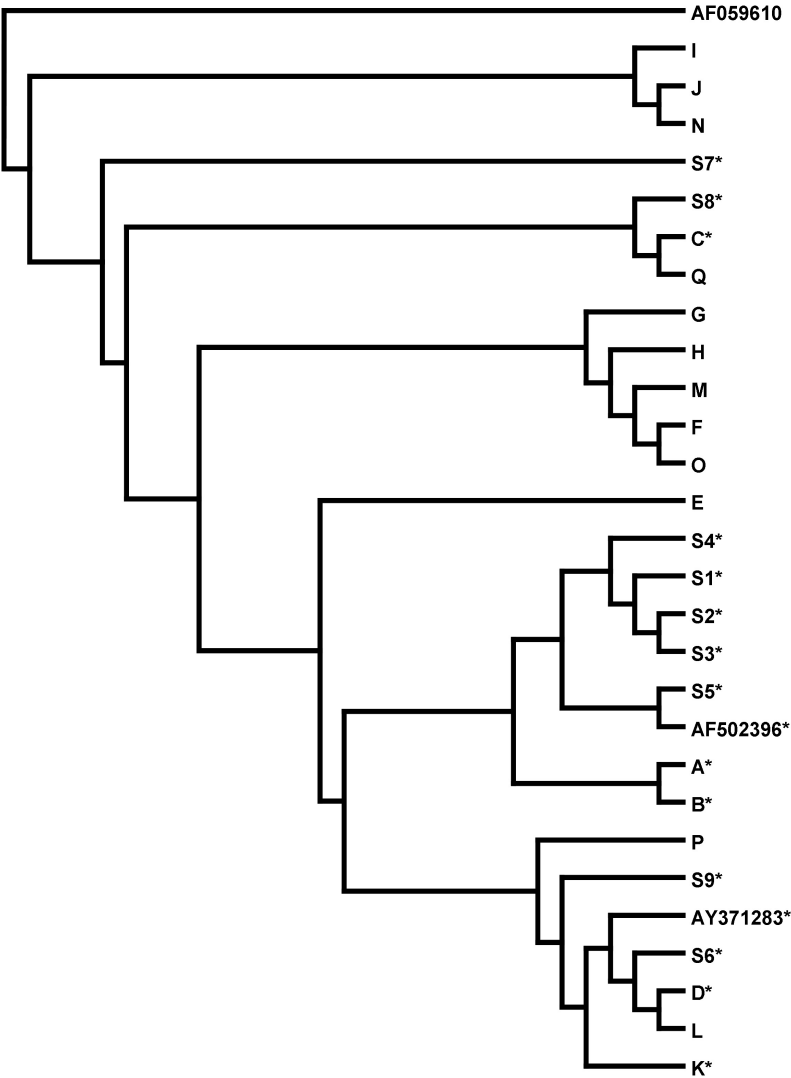


Figure 8.2. Most parsimonious tree based on the neighbor-joining analysis of the ITS region of the rRNA gene of *Enterocytozoon bieneusi* isolates. The genotypes derived from isolates in this study are in accentuated with an asterisk. The tree was defined with an outgroup taxon (AF059610).



DISCUSSION

More than 50 genotypes of *E. bieneusi* described, based on the ITS region, show a certain degree of host specificity (Mathis et al., 2005). In the present study, using 57 fecal-DNA samples in which *E. bieneusi* was detected by real-time PCR (Verweij et al., 2007c), 16 genotypes were identified of which nine showed novel variations fixed at 22 positions in the ITS sequence.

The increasing number of *E. bieneusi* genotypes thwarts systematic classification. Although the occurrence of polymorphisms at fixed base positions implies a stable divergence of the strains, the overall differences between the described genotypes are subtle. The interpretation of the phylogenic map based on a single locus has to be taken cautiously as this may not be representative for the genome as a whole (Anderson, 2001; Constantine, 2003). It can be argued that equal genotypes described in this study are formed by convergent evolution and if they can therefore be representative for the same genotypes described in previous publications. Genotypes presented on the phylogenetic map indicate a dynamic evolution of genotypes that do not always follow a linear path. Thus, analysis of at least a second, preferably transcribe gene should be used to determine the reliability of genotypes described on the basis of the ITS region.

Various host groups that have been described in the present study and in the literature have been associated with particular genotypes (table 8.3). We identified nine new genotypes (S1 – S9), genotypes A, B, C, AF502396, and AY371283, which were described already in previous studies in humans with and without HIV-infection (Rinder et al., 1997; Liguory et al., 1998; Breitenmoser et al., 1999; Tumwine et al., 2002), and genotypes D and K isolated from humans as well as from animals in different regions throughout the world (Dengjel et al., 2001; Sulaiman et al., 2003a). Both in Malawi as well as in The Netherlands, genotypes D and K have been represented most. Domestic animals were recognized as a possible source of transmission for genotypes D and K (Dengjel et al., 2001). The presence of genotypes D and K in different host species and its high representation in the present study might be the result of a combination of low transmission barriers between hosts and high virulence of the genotypes. In the phylogenetic map of figure 8.1, genotype K is shown as a common source for the further evolution of all other genotypes, with exception to genotype S7, which showed no linkage to any of the described genotypes. In the cladogram of figure 8.2, genotype S7 is related closest to the genotypes isolated from cattle. The patient with *E. bieneusi* genotype S7 infection was a middle-aged male presenting severe diarrhea and rectal bleeding. HIV infection was considered unlikely with this patient and except of diabetes, no other noticeable characteristics were recognized.

Of note is the detection of genotype C only in stool specimens of all five kidney transplant recipients tested. The predominance of this particular genotype with this particular patient group concurs with the previously described findings, in which seven of eight HIV-negative transplant recipients in France were also infected with genotype C (Liguory et al., 2001). The possibility of a common source of infection to explain this distribution of genotype C is unlikely as the material for these two studies was collected over a period of several years. Another similarity between the two studies is the increased prevalence of genotype B in HIV-infected patients both in France and in The Netherlands.

Individuals with a decreased immunity may show susceptibility to a wider variety of different parasite (sub)species since microorganisms tend to be less host specific in relation to hosts with a disturbed immunological barrier (Gatei et al., 2002; Chicharro & Alvar, 2003; Karp & Auwaerter, 2007). Probably this patient group is therefore also susceptible to a wider spectrum of different genotypes of *E. bienersi*. The number of different genotypes observed in HIV-positive adults in Malawi was higher than in The Netherlands. Nevertheless, the isolates originating from Malawi (i.e. genotypes AY371283, AF502396, D, K, S1, S2, S3, S4, S5 and S6) appear clustered while isolates originating from The Netherlands (i.e. genotypes A, B, C, D, K, S7, S8 and S9) are dispersed over a larger area of the phylogenetic map. Presumably, the genotypes found in patients from The Netherlands showed less homology because the clinical background of the patients with microsporidiosis is more diverse, whereas in Malawi the isolates originated mainly from HIV-positive persons. The cluster of genotypes from Malawi might also be the result of the ubiquity of spores in the environment. Age-dependant host specificity could not be demonstrated reliably in our study because of the incomplete sampling of patients: most patients in The Netherlands were adults while there were no immunocompetent adults from Malawi. The predominance of genotypes in specific populations can also be observed in animal hosts, where genotype clusters have been observed: genotypes E, G, F, M, O, and H described in pigs and genotypes J, I, and N in cattle (figure 8.1) (Dengjel et al., 2001; Sak et al., 2008).

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CHAPTER 9

MULTIPLEX REAL-TIME PCR FOR THE DETECTION AND QUANTIFICATION
OF *SCHISTOSOMA MANSONI* AND *S. HAEMATOBIIUM* INFECTION IN STOOL
SAMPLES COLLECTED IN NORTHERN SENEGAL.

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SUMMARY

A multiplex real-time PCR assay for the detection and quantification of *Schistosoma mansoni* and *S. haematobium* DNA in faecal samples was developed and evaluated as an alternative diagnostic method to study the epidemiology of schistosomiasis. Primers and probes targeting the cytochrome c oxidase gene were designed for species-specific amplification and were combined with an internal control. Using positive control DNA extracted from adult *Schistosoma* worms and negative control samples (n = 150) with DNA from a wide range of intestinal microorganisms, the method proved to be sensitive and 100% specific. For further evaluation, duplicate stool specimens with varying *S. mansoni* egg loads were collected in northern Senegal from pre-selected individuals (n = 88). The PCR cycle threshold values, reflecting parasite-specific DNA loads in faeces, showed significant correlation with microscopic egg counts both for *S. mansoni* in stool and *S. haematobium* in urine. The *Schistosoma* detection rate of PCR (84.1%) was similar to that of microscopy performed on duplicate stool samples (79.5%). The simple faecal sample collection procedure and the high throughput potential of the multiplex real-time PCR provide a powerful diagnostic tool for epidemiological studies on schistosomiasis in remote areas, with possibilities for extension to other helminths or protozoa using additional molecular targets.

INTRODUCTION

Worldwide, an estimated 200 million people are infected with *Schistosoma* spp., causing schistosomiasis or bilharzia (Gryseels et al., 2006). On the African continent the two most common species are *Schistosoma mansoni* and *S. haematobium*. Epidemiological studies on schistosomiasis traditionally rely on the detection of parasite eggs in stool (*S. mansoni*) or urine (*S. haematobium*) by microscopy. These methods are relatively inexpensive, easy to perform and provide information on prevalence and intensity of *Schistosoma* infections (Feldmeier & Poggensee, 1993). However, they also have a number of drawbacks. Samples need to be processed within 24–48 h and the processing itself can be rather tedious. Moreover, multiple sampling is needed to obtain an accurate impression of the presence and intensity of infection.

As an alternative for detecting *S. mansoni* infections, antigen-based assays, such as circulating cathodic antigen detection in urine, have proven to be a valuable, field-applicable method. Unfortunately, the test has been shown to be less sensitive for infections with *S. haematobium* (Van Dam et al., 2004). *Schistosoma*-specific antibody detection is considered to be highly sensitive, but this method cannot distinguish active from past infection. Blood collection is not easily applicable under field conditions, therefore this method is not recommended for field studies in endemic areas (Gryseels et al., 2006).

PCR-based methods have shown high sensitivity and specificity for the detection of parasitic DNA, yet their use in epidemiological surveys has so far been limited. For a long time, DNA isolation and subsequent DNA amplification was known to be laborious and expensive. However, recent developments in the simplification of DNA isolation procedures and PCR technology, especially real-time PCR, have made DNA amplification a worthy alternative to microscopy-based diagnostic methods (Klein, 2002; Verweij et al., 2004a, 2007b; Espy et al., 2006).

To date, conventional PCR methods for the detection of *Schistosoma* DNA in human samples have been published in which the rRNA gene [small subunit (SSU) rRNA], a highly repeated 121 bp sequence of *S. mansoni* or mitochondrial genes (*nad5*, *nad6* and *cox2*) were used as targets (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 targeting a small fragment of 96 bp on the SSU rRNA gene (Gomes et al., 2006). Although high sensitivity on control DNA was achieved, this real-time PCR only detected *S. mansoni* DNA and did not include an internal control.

The present study describes a real-time PCR for specific detection and quantification of *S. mansoni* DNA in faecal samples as an alternative to microscopy in

epidemiological research. Since eggs of *S. haematobium* can also be found in the rectal wall (Azar et al., 1958), a combined real-time PCR was developed to detect additionally *S. haematobium*-specific DNA in faeces using primers and probes targeting the cytochrome c oxidase subunit 1 (cox1) gene in the mitochondrial genome. The PCR assay was designed on the cox1 gene because the DNA sequences show sufficient divergence between separate *Schistosoma* species whilst a very low level of variation is expected within isolates collected in the same geographical region (Le et al., 2000; Morgan et al., 2005). Additionally, an internal control for the detection of possible inhibition of amplification by faecal contaminants was included in the assay.

The performance of the multiplex real-time PCR assay was evaluated using a range of DNA controls as well as stool samples collected in a remote area of northern Senegal, where both *S. mansoni* and *S. haematobium* are endemic.

MATERIALS AND METHODS

CONTROLS

Positive control DNA was extracted from adult *S. mansoni* and *S. haematobium* worms and eggs derived from infected hamsters as well as from human faecal samples confirmed positive for *S. mansoni* by microscopy.

The specificity of the multiplex PCR was tested using: (i) DNA isolated from individual adult worms of *Trichuris trichiura*, *Ascaris lumbricoides*, *Necator americanus*, *Opisthorchis felinus* and *Fasciola hepatica* and DNA of *Strongyloides stercoralis* first-stage larvae and *Ancylostoma duodenale* third-stage larvae. Furthermore, *Entamoeba histolytica*, *Entamoeba dispar*, *Dientamoeba fragilis*, *Giardia lamblia*, *Cryptosporidium parvum*, *Cyclospora cayentanensis*, *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis* DNA was tested in the PCR (Verweij et al., 2007c); (ii) DNA obtained from 15 different bacterial/yeast cultures of *Enterococcus faecalis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella enteritidis*, *Enterobacter aerogenes*, *Yersinia enterocolitica*, *Bacillus cereus*, *Shigella flexneri*, *Proteus mirabilis*, *Candida albicans*, *Salmonella typhimurium*, *Shigella dysenteriae*, *Shigella sonnei*, *Shigella boydii* and *Campylobacter upsaliensis*; and (iii) 80 DNA extracts from stool specimens that were positive by microscopy and confirmed by species-specific PCR for *E. histolytica* (n = 20), *E. dispar* (n = 20), *G. lamblia* (n = 20) and *C. parvum* (n = 20). The PCR assay was also tested on 40 unpreserved stool samples from individual patients with a negative result in microscopy using formalin–ether sediments and modified acid-fast staining as well as a negative *Giardia* antigen test. Two subsequent stool samples of these 40 patients also tested negative by microscopy and *Giardia* antigen test.

FIELD SAMPLES

For evaluation of the multiplex PCR, 176 stool samples from 88 subjects (age range 2–83 years, median 20 years; 58% female) were collected over a 2-week period in August 2006 in a village in northern Senegal. The village is endemic for both *S. mansoni* and *S. haematobium* (De Clercq et al., 1999) and no mass treatment programme with schistosomicides had taken place in the village in recent years. Participants were selected on delivery of at least two stool and two urine samples and representing a wide range of egg excretions based on microscopic analysis of the first stool sample. Within 24 h after production, faecal suspensions (congruent with 0.25 g/ml) were prepared in 70% ethanol. No major abnormalities, such as bloody appearance, were observed in stool consistency. Suspensions were stored and transported at room temperature until further processing was performed at the Laboratory for Parasitology, Leiden University Medical Centre, The Netherlands, within a couple of weeks after collection.

MICROSCOPY

For *S. mansoni*, egg excretion was determined by duplicate 25 mg Kato examinations on each stool sample (Katz et al., 1972). Intensities of infection were expressed as eggs per gram of faeces (EPG). For *S. haematobium*, eggs were quantified using the urine filtration method (Peters et al., 1976). Results were expressed as eggs per 10 ml of urine (EP10 ml).

DNA ISOLATION

For isolation of DNA, 250 µl of ethanol–faeces suspension was centrifuged. The pellet was washed twice with PBS and finally suspended in 200 µl of PBS containing 2% polyvinylpyrrolidone (Sigma, Steinheim, Germany). The suspension was heated for 10 min at 100 °C and following sodium dodecyl sulphate–proteinase K treatment (2 h at 55 °C) DNA was isolated using QIAamp Tissue Kit spin columns (QIAGEN, Hilden, Germany) (Verweij et al., 2001). Within the isolation lysis buffer, 103 plaque-forming units/ml of phocin herpes virus 1 (PhHV-1) was added to serve as an internal control (Niesters, 2002).

PCR AMPLIFICATION AND DETECTION

Schistosoma mansoni- and *S. haematobium*-specific PCR primers and detection probes were designed using Primer Express 2.0 (Applied Biosystems, Foster City, CA, USA) on the *cox1* sequence (GenBank accession numbers NC_002545 and AY157209, respectively). For detection of *S. mansoni*-specific DNA, primers Smcyt748F and Smcyt847R were selected to amplify a fragment of 99 bp, which is detected by the double-labelled probe Smcyt785T (Biolegio, Nijmegen, The Netherlands). For detection of *S. haematobium*-specific DNA, primers Sh307F and Sh447R were

selected to amplify a fragment of 143 bp, which is detected by the Minor Groove Binding (MGB) TaqMan probe Shaem377MGB (Applied Biosystems, Warrington, UK). For an internal control, the PhHV-1-specific primers and probe set consisted of forward primer PhHV-267s, reverse primer PhHV-337as and the specific double-labelled probe PhHV-305tq (Biolegio) (Niesters, 2002). All primers and detection probes are described in table 9.1.

Table 9.1. Oligonucleotide primers and detection probes for real-time PCR for the simultaneous detection of *Schistosoma mansoni* and *S. haematobium* as well as phocin herpes virus 1 (PhHV-1) as an internal control.

Target organism/oligonucleotide name	Oligonucleotide sequence	GenBank accession no. or literature reference of target sequence
<i>Schistosoma mansoni</i>		
Smcyt748F	5'-CCCTGCCAAATGAAGAGAAAAC-3'	NC_002545
Smcyt847R	5'-TGGGTGTGGAATTGGTTGAAC-3'	
Smcyt785T	FAM-5'-CCAAAACCAGACCCCTCTCAAATTG-3'-BHC1	
<i>Schistosoma haematobium</i>		
Sh307F	5'-CCTCCATTATCCATATCTGAGAATTCA-3'	AY157209
Sh447R	5'-AGAAGTCTTAAATCGACACGACTAATAATC-3'	
Shaem377MGB	NED-5'-ACCAACTAGTCTAGATACAC-3'-MGBNFQ	
PhHV-1		
PhHV-267s	5'-GGGCGAATCACAGATTGAATC-3'	Niesters, 2002
PhHV-337as	5'-GCGGTTCCAAACGTACCAA-3'	
PhHV-305tq	Cy5-5'-TTTTATGTGTCCGCCACCATCTGGATC-3'-BHQ2	

For amplification of DNA extracted from individual worms, eggs or stool specimens, 5 µl of eluted DNA was used as a template in a final volume of 25 µl with PCR buffer (HotStarTaq Master Mix; QIAGEN), 2.5 µg of bovine serum albumin (Roche Diagnostics Nederland B.V., Almere, The Netherlands), 5 mM MgCl₂, 2.0 pmol of each *S. mansoni* primer, 5.0 pmol of each *S. haematobium* primer, 3.75 pmol of each PhHV-1-specific primer, and 2.5 pmol of *S. mansoni*, *S. haematobium* and PhHV-1 detection probes. Amplification consisted of 15 min at 95 °C followed by 50 cycles of 15 s at 95 °C and 1 min at 60 °C. Amplification, detection and data analysis were performed with the Applied Biosystems 7500 Real Time PCR System and Sequence Detection Software version 1.2.2.

Serial 10-time dilutions of *S. mansoni* and *S. haematobium* control DNA were used to optimise the conditions for DNA amplification. Furthermore, each dilution series was

tested for cross-reaction between the two species and to assess the ability to detect mixed infections.

DATA ANALYSIS

Results of microscopy and real-time PCR analysis were stored in a Microsoft Access database (Microsoft, Redmond, WA, USA). For statistical analysis, data were entered in SPSS 11.0.1 (SPSS Inc., Chicago, IL, USA).

Intensity of infection as determined by the cycle threshold (Ct) value of the real-time PCR (reflecting faecal parasite-specific DNA loads) or microscopy (i.e. EPG or EP10ml) were described for the first and second stool and urine samples separately. Data were depicted as number, range and median value of positive subjects. Analysis agreement between the subject's outcome by real-time PCR analysis and microscopy was expressed using the κ statistic, considering a subject as positive for a diagnostic test when at least one of the duplicate stool or urine samples shows a positive outcome.

In addition, the mean number of egg counts of the subject's duplicate stool or urine samples was calculated as well as the mean Ct value of the two stool samples. For this purpose, negative values were redefined with a value representing an infection just below the detection limit of the diagnostic test used, i.e. one-half the detection limit in the case of microscopy and 3 Ct values above the highest detected Ct value for real-time PCR, i.e. a Ct of 42 and 49 for the *S. mansoni* and *S. haematobium* PCRs, respectively.

Spearman's non-parametric correlation coefficient (ρ) was used to calculate concordance in intensity of infection as determined by microscopy and real-time PCR, respectively. Statistical significance was considered at $P < 0.05$.

RESULTS

The real-time PCR was optimised first as a monoplex with 10-time dilution series of *S. mansoni* and *S. haematobium* DNA. Thereafter, monoplex real-time PCRs were compared with the multiplex PCR with *S. mansoni* and *S. haematobium* assays combined with PhHV-1 internal control. The Ct values obtained from testing the dilution series of *S. mansoni* and *S. haematobium* in the individual assays and the multiplex assays showed no systematic deviation in amplification curves, and the same analytical sensitivity was achieved. One hundred femtograms of both *S. mansoni* and *S. haematobium* DNA could be detected, also in the presence of 1 ng of DNA of the other *Schistosoma* species or internal control.

The specificity of the multiplex real-time PCR was evaluated using 150 DNA controls derived from a wide range of intestinal microorganisms as described in Section 2.1.

Amplification of *Schistosoma* DNA was not detected in any of these samples; only amplification of the internal control was detected at the expected Ct of approximately 33.

For a more elaborate evaluation of the multiplex real-time PCR, a selection of duplicate stool and urine samples from 88 subjects were used. Microscopic and PCR results are summarised in table 9.2. The numbers of subjects showing *S. mansoni* eggs in stool and *S. haematobium* eggs in urine were 70 (80%) and 63 (72%), respectively. In 49 subjects (56%) both *Schistosoma* species were detected, and 4 subjects (5%) showed no *Schistosoma* egg excretion. Amplification and detection of the PhHV-1 internal control was within the range of expected Ct values in all faecal samples (median 32.7). Specific amplification was detected for *S. mansoni* in 64 subjects (73%) and for *S. haematobium* in 48 subjects (55%). Specific amplification of both *S. mansoni* and *S. haematobium* was detected in 38 subjects (43%), and 14 subjects (16%) showed no DNA amplification for either of the two *Schistosoma* species. Statistical analysis showed a κ value of 0.44 between the detection of *S. mansoni* infection by microscopy and real-time PCR ($n = 88$; $P < 0.001$).

Table 9.2. Number (%) of positive cases and detected values (range and median of positive cases) of 88 subjects showing *Schistosoma mansoni* eggs in stool by Kato^a or *S. haematobium* eggs by urine filtration technique^b compared with the number and detected values of subjects showing *S. mansoni* or *S. haematobium* DNA in stool (Ct values) by real-time PCR.

Examination ^c	No. (%) of positive cases	Range	Median
1st Kato	64 (73)	20–5580	200
2nd Kato	53 (60)	20–9280	180
Both Katos	70 (80)	15–7080	128
1st urine	55 (63)	1–2180	14
2nd urine	48 (55)	1–596	23
Both urines	63 (72)	1–1093	17
1st <i>S. mansoni</i> PCR	61 (69)	21.1–38.5	27.1
2nd <i>S. mansoni</i> PCR	53 (60)	21.7–38.4	25.8
Both <i>S. mansoni</i> PCRs	64 (73)	21.9–39.5	27.6
1st <i>S. haematobium</i> PCR	32 (36)	28.9–45.9	37.2
2nd <i>S. haematobium</i> PCR	39 (44)	29.1–44.1	40.7
Both <i>S. haematobium</i> PCRs	48 (55)	29.0–47.5	41.2

^aEggs per gram of faeces.

^bEggs per 10 ml of urine.

^cStool and urine examination of first and second sample, and both samples, where a case is considered positive if in at least one of the two samples eggs or DNA were detected. For further explanation see the Materials and Methods section.

Table 9.3 shows the PCR results per *S. mansoni* egg count class. Discrepancies between the outcome of microscopy and PCR only occurred in subjects with very low egg counts or when no eggs were observed. *Schistosoma mansoni*-specific amplification was detected in all subjects with high, moderate and low *S. mansoni* egg counts. Additionally, *S. mansoni* PCR-positive samples showed a decreasing median Ct value with increasing egg count category, and an overall significant correlation was found between egg counts (EPG) and Ct values ($p = -0.77$; $n = 88$; $P < 0.001$).

Table 9.3. Comparison of *Schistosoma mansoni*- and *S. haematobium*-specific real-time PCR performed on DNA isolated from stool samples with Kato results for the detection of *S. mansoni* and with urine filtration results for the detection of *S. haematobium* from 88 subjects, categorised by number of eggs.

Egg count category ^a	N	No. (%) PCR +ve	Ct value ^b	
			Range	Median
<i>Schistosoma mansoni</i>				
No eggs	18	6 (33)	24.4–39.5	35.4
Very low (15–100 EPG)	32	20 (63)	25.0–38.0	33.1
Low (101–400 EPG)	16	16 (100)	24.5–38.3	27.1
Moderate (401–1000 EPG)	14	14 (100)	23.3–37.3	25.6
High (>1000 EPG)	8	8 (100)	21.9–27.2	23.4
Total	88	64 (73)	21.9–39.5	27.6
<i>S. haematobium</i>				
No eggs	25	7 (28)	43.3–47.5	45
Low (1–50 EP10 ml)	44	24 (55)	31.8–46.1	41.5
High (>50 EP10 ml)	19	17 (89)	29.0–46.6	37.8
Total	88	48 (55)	29.0–47.5	41.2

^a EPG: egg count per gram faeces; EP10 ml: egg count per 10 ml urine.

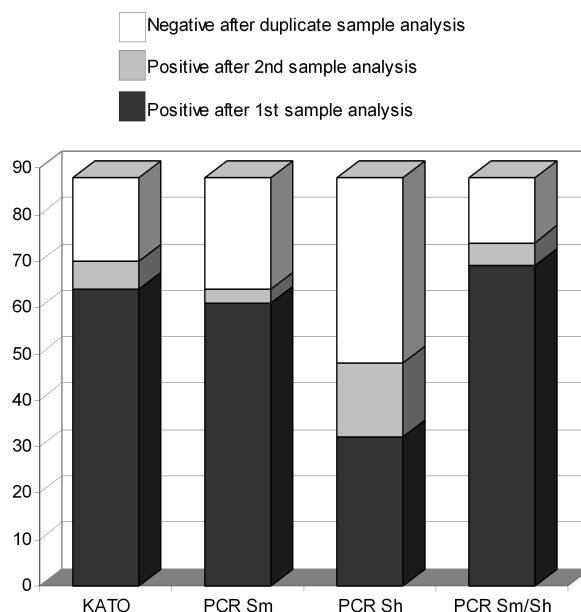
^b The range and median Ct value are calculated for positive PCR samples per egg count category.

A κ value of 0.31 was shown between the outcomes of microscopy performed on urine and *S. haematobium* DNA detection in stool ($n = 88$; $P < 0.005$). *S. haematobium*-specific amplification in faecal DNA samples was detected in 17 (89%) of 19 subjects in whom more than 50 *S. haematobium* EP10ml were detected (table 9.3). The discrepancy between real-time PCR and microscopy increased when fewer than 50 *S. haematobium* EP10ml urine were detected. Nevertheless, a decreasing median Ct value of *S. haematobium* PCR-positive samples is observed with increasing egg count categories, and Ct values as detected in the *S. haematobium*-specific real-time PCR showed significant correlation ($\rho = -0.59$; $n = 88$; $P < 0.001$) with the quantitative interpretation of eggs counted in urine.

Figure 9.1 shows the cumulative number of schistosomiasis positives by microscopy and PCR, respectively. After analysing the first stool series, 64 (72.7%) of 88 subjects were found to be positive by microscopy whereas 69 (78.4%) were found to be positive by PCR. Six more subjects (70/88; 79.5%) became positive after analysing

the second stool series by microscopy and five more (74/88; 84.1%) by PCR.

Figure 9.1. Cumulative number of subjects (n = 88) found to be positive for schistosomiasis after first and second analysis of stool samples by Kato or real-time PCR. Sm, *Schistosoma mansoni*; Sh, *S. haematobium*.



DISCUSSION

In this study, a multiplex real-time PCR assay for the detection of *S. mansoni* and *S. haematobium* was developed and evaluated using well defined DNA and stool samples as controls. In the pre-selected study population, 100% sensitivity of *S. mansoni* PCR was shown for subjects with on average more than 100 EPG detected in two stool samples, and a significant association was demonstrated between egg excretion and Ct values, representing the amount of parasite DNA in faeces. Discrepancies between microscopy and PCR analysis are observed both ways only in samples with very low egg counts or low parasite DNA concentration. Significant correlation between egg counts and Ct values was still achieved if only *S. mansoni*-specific PCR results of the first collected stool samples were included (data not shown), indicating that molecular analysis of a single stool sample already provides sufficient data. In this study, microscopy-positive samples were overrepresented. Therefore, a cross-sectional study is planned that would include a higher proportion of samples without detected eggs to obtain a more precise estimation of sensitivity.

Molecular-based diagnostic techniques for schistosomiasis have been described in previous reports (Pontes et al., 2002, 2003; Gomes et al., 2006; Sandoval et al., 2006). A valid comparison with these methods is difficult as they are all very diverse in their use of DNA targets, DNA isolation, PCR procedure and methods of sample selection.

Although eggs of *S. haematobium* were not observed in stool samples, the presence of *S. haematobium* DNA was detected successfully. As expected, the sensitivity of *S. haematobium* DNA detection in faecal samples is lower than the sensitivity achieved by microscopic examination of urine samples. A preliminary study using the real-time PCR on DNA isolated from urine samples, collected in a *S. haematobium*-endemic area in Gabon, already shows promising results (unpublished data).

Obviously, because of the relative high costs, this PCR procedure is not intended for routine diagnosis in clinical settings in *Schistosoma*-endemic countries. On the other hand, prices of equipment and consumables are becoming more and more attractive. This trend, in combination with rapid improvements in technical performances, has already resulted in an increasing number of centrally located research centres within low income countries having real-time PCR technology available, for example for HIV viral load determination. In our opinion, the described *Schistosoma* PCR provides a powerful alternative or additional diagnostic tool for these research centres. The use of ethanol-suspended stool samples for storage and transport at room temperature makes collection of samples at remote areas more attractive. This has already been used successfully for hookworm multiplex real-time PCR, where microscopic egg or larva counts were also reflected by the Ct values (Verweij et al., 2007a). This also illustrates that the procedure can easily be extended towards real-time PCR assays for the detection of a whole range of intestinal microorganisms, such as *G. lamblia*, *Cryptosporidium* sp., *E. histolytica* or microsporidia (Verweij et al., 2004a, 2007c; Ten Hove et al., 2007).

In conclusion, the multiplex real-time PCR for the detection of *S. mansoni* and *S. haematobium* has been shown to be a powerful tool for epidemiological studies and can provide additional value in the evaluation of control programmes for the following reasons: (i) possible storage of stool samples at room temperature without need for direct sample processing; (ii) high throughput potential; (iii) quantitative output; (iv) the combination of multiple molecular targets and internal control in one assay; and (v) the possibility of using the isolated DNA for targeting additional intestinal microorganisms.

ETHICAL APPROVAL

This study is part of a larger investigation on schistosomiasis epidemiology and

control in Senegal, for which approval was obtained from the ethical committees of the Institute of Tropical Medicine in Antwerp, Belgium, and the Ministry of Health in Dakar, Senegal.

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CHAPTER 10

SCHISTOSOMIASIS PCR IN VAGINAL LAVAGE AS AN INDICATOR OF GENITAL

SCHISTOSOMA HAEMATOBIMUM INFECTION IN RURAL ZIMBABWEAN

WOMEN

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ABSTRACT

Schistosoma real-time PCR is sensitive and specific in urine and stool. We sought to explore the relationship between genital schistosomiasis and the *Schistosoma* PCR in women. PCR was run on 83 vaginal lavage samples from a rural Zimbabwean population. Women underwent clinical and colposcopic investigations, analyses for sexually transmitted infections and genital schistosomiasis. Thirty samples were positive for *Schistosoma* PCR: 12 were strong and 18 were weak positive. Sensitivity and specificity were best in women below the age of 25 years. *Schistosoma* PCR was associated with *S. haematobium* ova in genital tissue, so-called sandy patches and bleeding. PCR prevalences were lower and Ct real-time PCR values weaker in older women. The presence of *Schistosoma* DNA may be greater in the younger recent lesions, eg, in younger women. In rural and mass research *Schistosoma* PCR could become a supplement to or even replace gynaecologic examinations.

INTRODUCTION

Seven hundred million people are at risk of acquiring schistosomiasis, most of them in Africa. (WHO, 2002). Up to 75% of the women excreting *S. haematobium* eggs in urine have been found also to have schistosome eggs in the uterine cervix, vagina or vulva (Renaud et al., 1989; Kjetland et al., 1996; Poggensee et al., 2000; Fenwick, 2006). Furthermore women may have genital schistosomiasis without urinary findings. Genital schistosomiasis is associated with at least four genital mucosal manifestations, namely two types of sandy patches, abnormality in blood vessels and contact bleeding (Gibson et al., 1925; Badawy et al., 1962; Poggensee et al., 1998; Kjetland et al., 2005). The disease may cause discharge, genital itch, spot bleeding and possibly also infertility and dyspareunia (Poggensee et al., 2000; Kjetland et al., 2008). It has been suggested that schistosomiasis could be responsible for increased susceptibility to HIV, and a study in Zimbabwe reported a 3-fold higher odds ratio for HIV seropositivity in women with genital schistosomiasis, compared to those without (Feldmeier et al., 1994; Chenine et al., 2008; Kjetland et al., 2006). This raises the possibility that treatment and prevention of genital schistosomiasis may have an impact on HIV transmission in areas where both infections are prevalent. Improvements in the detection of genital schistosomiasis would be an important component of such a strategy.

Currently female genital schistosomiasis is diagnosed by visual inspection of the cervicovaginal mucosa and on the microscopic detection of parasite eggs in biopsies, wet smears or Papanicolau (Pap) smears (Poggensee et al., 2001; Kjetland et al., 2005). These investigations have limitations in resource-poor settings – they may be cumbersome and expensive, require rural electricity, water, and both clinical and laboratory equipment. The current gold standard for diagnosis is a biopsy (Poggensee et al., 2001). However, the inflicted wound from a biopsy could pose a risk for HIV transmission for the patient or her partner and should therefore not be performed in routine investigations (Wright et al., 2001; Kjetland et al., 2005).

Multiplex real-time PCR, including an internal control for the detection of inhibiting factors, has been described for the detection of *Schistosoma mansoni* and *S. haematobium* DNA in stool or urine samples as an alternative for microscopic detection of parasite ova (Ten Hove et al., 2008; Obeng et al., 2008). These PCRs have shown 100% specificity. Furthermore, the semi-quantitative outcomes of real-time PCRs defined by Cycle threshold (Ct)-values are significantly correlated with the microscopic egg counts in faeces and urine. In subjects excreting more than 50 eggs per 10ml of urine a sensitivity of 100% has been described using the internal-transcribed-spacer-2 (ITS-2) based *Schistosoma*-genus-specific real-time PCR (*Schistosoma* PCR) in urine samples (Obeng et al., 2008). In this paper we report

Schistosoma PCR in vaginal lavage material for the diagnosis for the diagnosis of female genital schistosomiasis.

MATERIALS AND METHODS

STUDY SUBJECTS AND AREA.

The study was conducted from October 1998 to March 1999 in Mupfure area in Mashonaland Central Province in Zimbabwe and has been described in detail previously (Kjetland et al., 2005). Briefly local village health workers listed female household members in the area they assumed were sexually active (based on personal judgment). All listed women aged 15-49 years resident in the indicated area were invited to take part in the study. From the area directly around the clinic and at 3 pick-up points 83% attended, whereas there was a 33% attendance from the surrounding areas. Groups were comparable in waterbody contact, sexually transmitted diseases and marital status. Virgins, pregnant, postmenopausal or menstruating women, those who refused to undergo gynaecological examination or participate and those who had other serious diseases were excluded.

ETHICAL CONSIDERATIONS.

Inclusion in the study took place after individual informed oral consent. In addition permission was given by the Provincial and District Medical Directors and by the village headman at village meetings. Ethical approval was given by the Medical Research Council of Zimbabwe (MRCZ/A/578) and by the ethical committee of the Special Programme for Research and Training in Tropical Diseases Research (TDR), UNDP/WB/ WHO provided that biopsies were taken only upon an unconfirmed suspicion of malignancy since lesions may pose an entry port for HIV and many women in the area may not decide whether to have intercourse (Hindin, 2003).

All included and excluded patients found positive for urinary or genital schistosomiasis were treated with praziquantel 40mg/ kg in one dose (Lessing, 1998; Kjetland et al., 2005). Patients with symptoms or signs of sexually transmitted infections (STI) or other diseases were treated in accordance with the Zimbabwean standard syndromic approach and referred to tertiary institutions when necessary (Lessing, 1998). Partner-treatment-slips were given to the index STI case; these ensured free treatment.

CLINICAL EXAMINATIONS AND SAMPLING.

At the time of the examination, the clinician did not know the result of *S. haematobium* examination in urine. Patients were interviewed in Shona (the local language). Examination was commenced with cervicovaginal lavage collected by spraying saline (5 ml) on the vaginal wall and cervix twice, whereupon it was drawn

back into a long syringe, from the posterior fornix, and deposited into 4 tubes and stored at -20 Degrees Centigrade. Subsequently photocolposcopic examination (Leisegang Photocolposcope, Script-O-Flash, Germany, Magnifications 7.5; 15; 30) was performed using autoclaved equipment. The mucosal and vulval surfaces were inspected section by section according to a predefined protocol. Homogenous yellow sandy patches were defined as sandy looking areas with no distinct grains using 15-times magnification. Grainy sandy patches constituted grains (app. 0.05 by 0.2 mm) situated in the mucosa. Neo-vascularisation was defined as pathological convoluted (cork-screw), reticular, circular and/ or branched, uneven-calibre blood vessels visible (by 15 times magnification) on the mucosal surface. Female genital schistosomiasis (FGS) is defined as the presence of sandy patches and / or ova in genital tissue. Contact bleeding was defined as fresh blood originating from the mucosal surface. Pre-contact bleeding was defined as darkened blood on the mucosal surface in the absence of recent or present menstruation. As reported previously some of the clinical phenomena were also associated with other diseases, e.g. homogenous yellow sandy patches were also associated with herpes simplex virus type-2 (HSV-2) and neovascularisation was associated with cervical intraepithelial neoplasia (Kjetland et al., 2005). Pap smears were taken from all consenting women with a wooden spatula. Wet mounts were taken from consenting women who showed debris or friable, loose and bleeding tissue with a titan autoclaved spatula (Swart & van der Merwe, 1987). Punch biopsies were taken only from consenting women when there was suspicion of malignancy. The investigation was finalised by bimanual examination.

Urine samples were collected on three consecutive days and 10 ml of each sample was processed by filtration for microscope examination for *Schistosoma* ova (Doehring et al., 1983). Single stool samples were collected from women and processed by Kato technique for the detection of *S. mansoni* ova (Katz et al., 1972). Finally, a venous blood specimen was collected. Serum was separated by centrifugation and stored at -20 Degrees Centigrade immediately after collection (Baay et al., 2004; Kjetland et al., 2005). Slides and tubes were not recycled.

ANALYSIS OF GENITAL SAMPLES

When there was an adequate amount of remaining sample, laboratory analysis for STIs were performed. Women were tested for a median of 7 STIs (range 1-8). Vaginal lavages samples were used for preparation of Gram-stained smears for detection *Candida* and for bacterial vaginosis, with the latter defined using Nugent's criteria (Joesoef et al., 1991). Pap smears were investigated for cell atypia and *S. haematobium* ova. Collected biopsies were each divided into two sections; one section was sent for histology and the other was retained to make a crushed biopsy and examined microscopically for ova (Feldmeier et al., 1981; Poggensee et al., 2001;

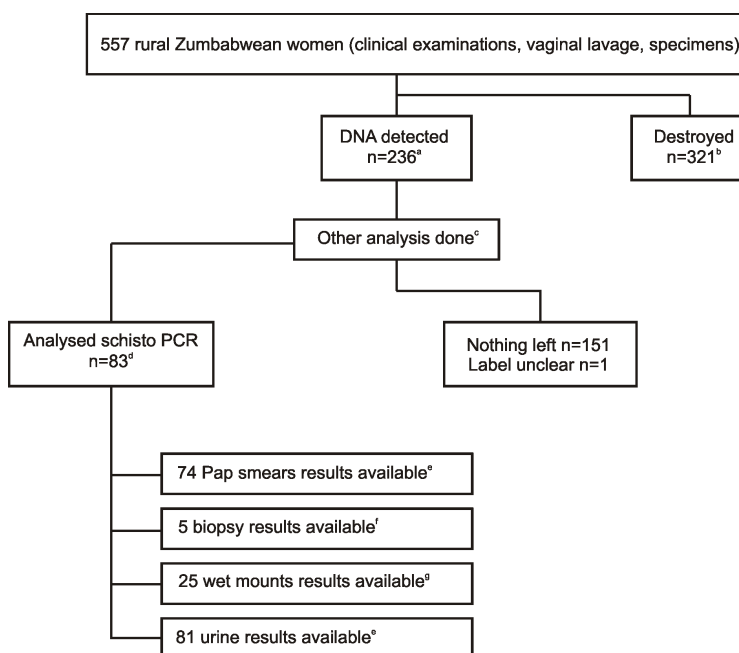
Kjetland et al., 2005). Wet smears were inspected by the field laboratory technician and by the clinician. Biopsies were explored by 2 independent pathologists and by the field clinician. Pap smears were inspected by an independent cytologist, by the field laboratory technician and the clinician.

POLYMERASE CHAIN REACTION ON GENITAL SAMPLES

Specimens were collected from 557 rural Zimbabwean women. DNA samples from 83 vaginal lavage specimens (15%) were available for *Schistosoma* real-time PCR analyses. The rest were excluded after a number of non-systematic, arbitrary events which have been described previously (Figure 10.1) (Baay et al., 2004). DNA was isolated from sediment of centrifuged vaginal lavage with the Wizard Genomic DNA Purification kit (Promega, Leiden, The Netherlands), according the manufacturer's protocol. The presence of DNA was confirmed by β -globin PCR using primers PC03/04 (Saiki et al., 1988). PCR was used to detect *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Haemophilus ducreyi* and human papillomavirus (Baay et al., 2004).

Ten years after the initial sampling in Zimbabwe, after the breakdown of 2 freezers under the fluctuating electricity and after a number of other analyses, real-time PCR for *Schistosoma* genus was performed on the remaining specimens (Baay et al., 2004; Obeng et al., 2008). Primers Ssp48F (5'-GGT CTA GAT GAC TTG ATY GAG ATG CT-3') and Ssp124R (5'-TCC CGA GCG YGT ATA ATG TCA TTA-3') were used to amplify a 77-bp fragment of the internal transcription spacer (ITS) 2 (homologous to both *S. haematobium* and *S. mansoni*), which was detected by the double labelled probe Ssp78T (FAM-5'-TGG GTT GTG CTC GAG TCG TGGC-3'-Black Hole Quencher) (Biolegio, Malden, The Netherlands). For detection of inhibitory effects during the amplification process, DNA of PhHV was added to the PCR mixture and detected with PhHV-1 specific primers and the specific double labelled probe (Biolegio) (Niesters, 2002). Species specific differentiation between *S. haematobium* and *S. mansoni* were made with the PCR targeting the cytochrome c oxidase (*cox1*) gene (ten Hove, 2008). Amplification, detection and data analysis were performed with the Applied Biosystems 7500 Real-Time PCR system and Sequence Detection Software version 1.2.2.

Figure 10.1. Recruitment of cases for *Schistosoma* real-time PCR analysis in rural Zimbabwean women.



^aTested by β -globin PCR using primers PC03/04. ^bBreakdown of two freezers under electricity fluctuations in Zimbabwe (Baay et al., 2004). ^cHuman papillomavirus PCR. ^d*Schistosoma* PCR detection (Obeng et al., 2008). ^eDone in all consenting women, some results were lost due to mistakes on / destruction of forms. ^fDone on suspicion of malignancy only. ^gDone in the presence of mucosal bleeding only.

SEROLOGY

Serology was used to detect antibodies against herpes simplex virus type 2 (HSV-2), *Trichomonas vaginalis* and *Treponema pallidum* (Rapid Plasma Reagin and *Treponema pallidum* haemagglutination assay). Active syphilis was defined as the presence of an ulcer and seroconversion during follow-up and/or a positive IgM serological test. All sera were tested for HIV with two test kits; a third kit was used in the event of discrepant results (Recombigen, Cambridge Biotech, Galway, Ireland; Genelavia Mixt, Sanofi Diagnostics Pasteur S.A., Marnes La Coquette, France; Vironostica HIV, Organon Technika, Boxtel, The Netherlands).

STATISTICAL METHODS

Chi-square, Fisher's exact tests (for numbers below 5) and odds ratio (OR) with 95% confidence interval were used when studying the association between laboratory results and clinical pathology in the genitals. In order to study simultaneously the impact of several variables, logistic regression analysis was applied on variables with a 20% significance level in bivariate analysis. The variable was excluded from multivariate analyses if they constituted less than 10 cases in denominator or numerator. Semi-quantitative *Schistosoma* real-time PCR results were categorised as strong ($Ct \leq 30$), weak ($Ct > 30$ and ≤ 50) or negative (undetected Ct -value redefined as 50). Sensitivity and specificity of the PCR were calculated using the combined results of the clinical and parasitological findings as gold standard. The diagnostic values of the available techniques were compared. Conversely, the PCR was used as a gold standard calculating the sensitivity and specificity of the individual field techniques. The statistical analysis was computed using SPSS (Statistical Package for the Social Sciences) version 14.0 and EpiInfo2000 (CDC, Atlanta, GA).

RESULTS

Table 1 shows that PCR-tested and untested women had similar levels of urinary and genital schistosomiasis, current water contact and HIV infection status. The mean age of the tested group was 2 years younger than the untested.

Table 10.1. Comparing characteristics of women who were and were not tested by *Schistosoma* real-time PCR for genital schistosomiasis.

	Schistosomiasis PCR ^a tested n (%)	Not tested by PCR n (%)	P
Urinary schistosomiasis ^b	37 / 81 (46)	202 / 469 (43)	0.66
Urine mean egg count/10ml (SD) ^c	8,4 (28,9)	3,6 (16,2)	0.28
Current regular use of river	76 / 83 (92)	413 / 473 (87)	0.25
Genital sandy patches ^d	37 / 83 (45)	219 / 474 (46)	0.78
HIV status	20 / 83 (24)	139 / 470 (30)	0.30
Mean body mass index (SD) ^e	21,9 (2,9)	22,2 (3,4)	0.74
Shona tribe ^f	73 / 83 (88)	433 / 472 (92)	0.28
Married	72 / 83 (87)	392 / 473 (83)	0.37
Mean age (SD)	31 (9)	33 (9)	0.034

^a*Schistosoma* real-time PCR (Obeng et al., 2008). ^bFrom all consenting women, some results were lost due to mistakes on forms, three consecutive urines. ^cOnly 4 cases had more than 50 ova/10ml urine. SD=standard deviation. ^dThe commonest current genital lesion in this population, caused by *S. haematobium*. ^eCalculated by dividing weight in kg by height in metres squared. ^fThe main population group in the area, others were from Matabeleland, Mozambique and Malawi.

In 83 samples, 30 (36.1%) were positive for *Schistosoma* real-time PCR: 12 samples were strong positive Ct values (range: 23.0 – 30.0; median 25.5) and 18 were weak positive (range 32.1 – 39.6; median 36.4). Table 2 shows that presence of *Schistosoma* DNA was significantly associated with sandy patches and *S. haematobium* ova in genital specimens. Some, but not all of the homogenous yellow and grainy sandy patch types seemed to contained *Schistosoma* DNA. Of the 37 women with homogenous yellow sandy patches 20 (54%) were found to have *Schistosoma* DNA, likewise *Schistosoma* DNA was found in 10/19 (52%) with grainy sandy patches and 17/32 (53%) of those with neovascularisation. Eleven of the 12 vaginal lavage positive with strong Ct values were found to have sandy patches. The presence of *Schistosoma* DNA was also associated with both neovascularisation and contact bleeding. The presence of *Schistosoma* DNA was also associated with both neovascularisation and contact bleeding. However *Schistosoma* DNA was not associated with polyps, ulcers and erosions, leukoplakia and papillomatous tumours, together or separate.

When adjusted for HSV-2 and age, homogenous yellow sandy patches remained associated with *Schistosoma* DNA (adjusted odds ratio with 95% confidence interval, Adj. OR 3.1, 95% CI (1.48-6.51), $p=0.003$). Likewise, neovascularisation was associated with *Schistosoma* DNA after adjusting for cervical intraepithelial neoplasia, HSV-2 and age (Adj. OR 3.1, 95% CI (1.5-6.4), $p=0.002$). After adjusting for high-risk human papillomavirus, HSV-2 and age, contact bleeding *Schistosoma* DNA was no longer significantly associated with contact bleeding *Schistosoma* DNA (Adj. OR 1.84, 95% CI (0.91-3.73), $p=0.091$). Grainy sandy patches were not associated with any other reproductive tract diseases, therefore no multivariate analysis was run for this clinical finding⁴. None of the other findings in Table 2 associated with the *Schistosoma* real-time PCR results were affected after adjusting for age (data not shown).

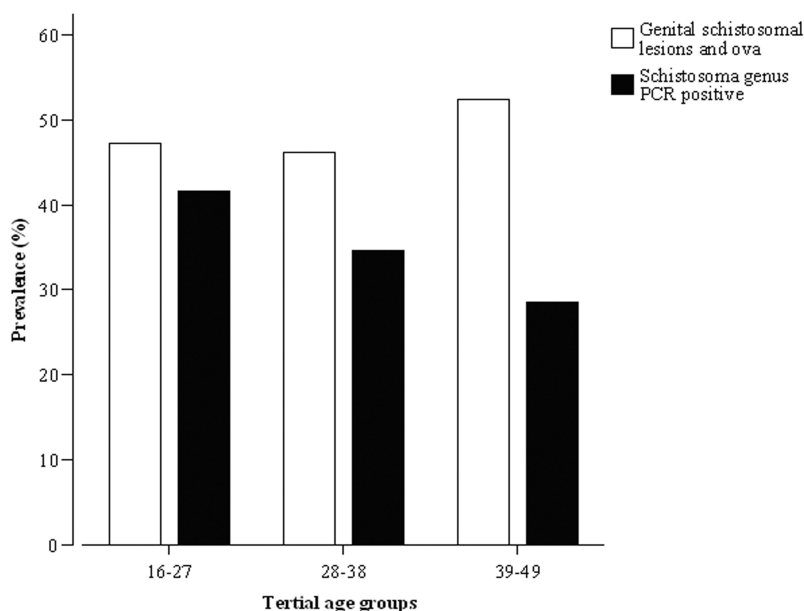
Table 10.2. *Schistosoma* PCR results association with gynaecological findings and urinary schistosomiasis in rural Zimbabwean women.

	<i>Schistosoma</i> real-time PCR ^a			p ^b
	High (n=12)	Low (n=18)	Negative (n=53)	
All sandy patch types (below) (n= 37)	11	9	17	<0,001
Homogenous yellow sandy patches ^c (n= 30)	10	7	13	<0,001
Both grainy sandy patch types ^d (n= 19)	5)	5	9	0.057
Superficial grainy sandy patch ^e (n= 9)	3	2	4	0.100
Deep grainy sandy patch ^f (n= 12)	3	3	6	0.216
Other clinical findings				
Mucosal neovascularisation ^g (n= 32)	8	9	15	0.007
Mucosal contact bleeding ^h (n= 24)	8	3	13	0.025
Mucosal pre-contact bleeding ⁱ (n= 14)	5	2	7	0.051
Mucosal oedema (n= 11)	5	1	5	0.017
Polypous tumour ^j (n= 1)	0	0	1	0.490
Leukoplakia or papillomatous tumour ^k (n= 8)	0	2	6	0.220
Erosion or ulcer ^k (n= 11)	2	0	9	0.490
<i>S. haematobium</i> in any genital specimen (below)^l (n=17)	7	3 / 17	7 / 49	0.003
<i>S. haematobium</i> ova in Pap smear ^m (n=6)	4	1 / 17	1 / 45	0.001
<i>S. haematobium</i> ova wet smear ⁿ (n= 9)	3 / 8	2 / 4	4 / 13	0.720
<i>S. haematobium</i> ova in biopsy ^o (n= 3)	1 / 1	0	2 / 5	0.840
<i>S. haematobium</i> ova in urine ^p (n= 37)	9	8 / 17	20 / 52	0.029
HIV seropositivity (n= 20)	3	4	13	0.97

^a*Schistosoma* genus detection by real-time PCR, high: Cycle threshold (Ct) < 30, low: Ct 30 - 50, negative: Ct >50. ^bLikelihood ratio. ^cHomogenous yellow sandy patches were sandy looking areas with no distinct grains using 15-times magnification. ^dBoth deep and superficial oblong grains (app. 0.05 by 0.2 mm) situated in the mucosa. ^eCould be felt with a metal spatula / bimanual palpation. ^fCovered with smooth mucosal membrane, seem to be deeper. ^gConvuluted (cork-screw), reticular, circular and/ or branched, uneven-caliber blood vessels. ^hFresh, red intravaginal blood originating from mucosal surface after careful insertion of speculum. ⁱDarkened intravaginal blood, not caused by recent menstruation. ^jSmooth pedunculated tumor originating from the cervix or vaginal walls. ^kAlso analysed separately (no difference found). ^l*S. mansoni* was not found in genital specimens. ^mpapanicolaou smears were done in all consenting women, some results were lost due to mistakes on / destruction of forms, 74 cases. ⁿDone on 25 cases in the presence of mucosal bleeding only. ^pTaken from 81 cases, some results were lost due to mistakes on / destruction of forms.

Seven cases were negative by *Schistosoma* real-time PCR even if *S. haematobium* ova were found in the genital specimens with microscopy (Table 2). Their ages were evenly distributed from 18 to 46 years of age and all had current waterbody contact. Five of these 7 cases each had at least one of the sandy patch types and 3 of these also had urinary schistosomiasis. The two that did not have sandy patches were found to have ova by biopsy; both had childhood water contact, lived in the area and had never been treated, one had urinary schistosomiasis and the other had neovascularisation and contact bleeding. Sandy patches were found in 17 *Schistosoma* PCR negative cases. All but one were using the river currently, and the last had waterbody contact just 6 months prior, as well as during childhood. *Schistosoma* genus real-time PCR had a 70% positive predictive value (PPV) for genital schistosomiasis, a sensitivity of 53% and a specificity of 79%. In the young women, below the age of 25, the values were slightly better: PPV was 77%, sensitivity 67% and specificity 83%. Younger women had stronger Ct values (data not shown). Figure 2 shows that the prevalences of *Schistosoma* genus PCR decrease with age even though the clinical findings were equally prevalent in all age groups.

Figure 10.2. Age distribution of female genital schistosomiasis and *Schistosoma* genus PCR positive cases.



Female genital schistosomiasis was equally present in all age groups whereas *Schistosoma* PCR decreases with age (Kjetland et al., 2005).

S. mansoni ova were found in 14 / 83 (17%) stool samples, mean intensity 4 eggs/g (SD=5). No *S. mansoni* was found in genital specimens. However *S. mansoni* DNA (*cox1*-based) was detected in one vaginal lavage sample, showing a weak Ct signal of 42. Likewise, *S. haematobium* DNA (*cox1*-based) was detected only in 18 of the 30 vaginal lavages with detectable *Schistosoma* genus DNA (ITS-2 based) (data not shown). The *cox1*-based *Schistosoma* PCRs had a lower sensitivity than the *Schistosoma* genus PCR (data not shown).

DISCUSSION

The presence of *Schistosoma* DNA *Schistosoma* real-time PCR is highly associated with the genital mucosal sandy patch types, neovascularisation and contact bleeding, also after correction for age and other diseases. Furthermore, as has been indicated previously, ulcers, polyps, papillomatous tumours and leukoplakia were not associated with *Schistosoma* real-time PCR (Kjetland et al., 2005). These data indicate that the presence of genital *Schistosoma* DNA decreases with age in adult women, contrary to previous findings where genital schistosomiasis prevalence is the

same in the different age groups (Poggensee et al., 2000; Kjetland et al., 2005).

The results of the sensitivity and specificity of *Schistosoma* real-time PCR in vaginal lavage may shed some light on the pathogenesis of FGS, and the sampling methods. Although better in detecting genital schistosomiasis than the other (ethically acceptable) laboratory tests the *Schistosoma* PCR sensitivity was lower than 60%. False negative *Schistosoma* real-time PCR in lavage may be attributed to the non-invasive nature of the lavage compared to the other genital tests. Pap smears, wet smears and biopsies harvest ova from the epithelium and deeper in the stroma and are, in order of appearance, more invasive than lavage (Poggensee et al., 2001; Kjetland et al., 2005). The vaginal lavage itself was not examined microscopically microscopically directly for *S. haematobium* ova, consequently the lavage analysis is fundamentally different from tests of faeces and urine. Moreover the histopathological studies have shown that ova may be situated deeply in genital tissue, in stroma, rather than in epithelium, as was the case in this population as well (Poggensee et al., 2001; Kjetland et al., 2005). *Schistosoma* DNA is not necessarily found in lavage in persons with deeply situated ova. Furthermore, we cannot preclude that local bleeding or foreign material, such as intravaginal water, soaps, herbs and sperm may dilute and disturb the *Schistosoma* PCR test giving false negative results (Kjetland et al., 2005). False positive cases may be caused by the fact that *S. haematobium* ova can also be found in sperm and history of sexual intercourse was not recorded in this study (Leutscher et al., 2005). The contamination from the outer genitals by urine or contamination from previous analyses (e.g. the practice of recycling/reusing slides/filters) was not an issue in this study.

The infrequent case reports of genital *S. mansoni* infections, compared to those from *S. haematobium* has been taken as an indication that *S. mansoni* rarely causes genital lesions in women (Richter, 2003; Oliveira et al., 2006). In this rural Zimbabwean population 17% were found to have *S. mansoni* in faeces, however, no *mansoni* ova were found in the genital specimens (Kjetland et al., 2005). We cannot, however, preclude that some of the positive PCRs represent *S. mansoni*.

Previous publications have shown higher sensitivities and specificities in stool and urine (ten Hove, 2008; Obeng et al., 2008). The urine report is from school children, and the stool report is from young adults (median age 20 years), whereas the median age was 33 years in this population. The current findings indicate that lesions, even superficial grains and inflammation, are found in the absence of *Schistosoma* DNA. However, *Schistosoma* prevalences were lower, Ct values weaker in older women, and the positive predictive value of the test was better in the young. This may indicate that there may be more DNA in the younger age groups, irrespective of lesion size. Furthermore the pathogenesis of the disease is not fully understood

(Smith & Christie, 1986; Kjetland et al., 2008). Viable ova may be found for a few weeks after parasite death but it is unknown for how long schistosome DNA persists. Previous reports suggest that genital schistosomal lesions are refractory to treatment (whereas urine ova excretion ceased). This may indicate that lesions do not heal even if ova have died or disintegrated. Moreover, several histopathological reports indicate that there may be inflammatory reaction around dead ova, presumably PCR negative, yet continuing to pose a clinical problem (Berry, 1976; Helling-Giese et al., 1996).

It has been suggested that young women should be treated in order to prevent adult lesions and HIV transmission (Kjetland et al., 2006). The timing and frequency of such treatment has not yet been explored. Treatment of other reproductive tract diseases may decrease genital HIV-RNA shedding, but this has not been explored for genital schistosomiasis (Orroth et al., 2000; Mayaud et al., 2001; Cohen et al., 2005; Sheffield et al., 2007; Kaul et al., 2008). Repeated anti-schistosomal mass treatment has been done in several countries and the effects are well-documented in the urinary tract (Fenwick, 2006). However, there is a paucity of documentation on the genital tract. One report indicates that there is a partial prevention of genital tract morbidity in those that were treated as teenagers (Kjetland et al., 2008).

Genital schistosomiasis lesions are often not visible to the naked eye and the investigations are subject to inter- and intra-observer variation and the quality of the equipment (Kjetland et al., 2005) (personal communication, Dr. Bodo Randrianasolo, Madagascar). Adequate gynaecologic investigations can be near to impossible in many rural areas. Many women are not accustomed to gynaecological investigations; the clinic rooms may be inadequately designed for privacy or lighted conditions, the waiting queues may be prohibitive for thorough inspections, electricity supply may be erratic and the turning of the speculum for inspection of the entire mucosa may be uncomfortable (Kjetland et al., 2005; Feldmeier et al., 1995).

For the pending research projects the *Schistosoma* real-time PCR could become a supplement to or even replace of gynaecologic examinations. PCR machines are now available in many university hospitals in endemic areas, rural and mass investigations could hence be limited to intravaginal swabs (Kjetland et al., 2005). Furthermore, self-sampling for the diagnosis of human papillomavirus PCR has already been tested in several settings (Baay et al., 2004; Serwadda et al., 1999; Wright et al., 2000). Although it is currently unrealistic to use *Schistosoma* PCR of vaginal lavage in ordinary clinical work in endemic areas, this is a method that could be put in evaluation of treatment and research.

These new *Schistosoma* real-time PCR analyses seem to corroborate that lesions may become chronic after deposition and death of ova. Furthermore, the presence of *Schistosoma* DNA in the young indicates that this method may be used to follow

them for effect of treatment. Further refining of the sampling methods and the use of intravaginal substances as a confounder should be explored. Female genital schistosomiasis as a cause of disease, infertility and risk for HIV could be explored along with the effect of treatment. Likewise, the issues of *S. mansoni* and male genital schistosomiasis as risk factors for HIV transmission to women are pending. The *Schistosoma* real-time PCR is a good candidate for diagnosis in research and evaluation projects, maybe especially in the young.

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CHAPTER 11

GENERAL DISCUSSION

MOLECULAR DIAGNOSIS OF INTESTINAL PARASITES

Within the fields of routine diagnostic virology and bacteriology real-time PCR has been established as a sensitive and specific qualitative and quantitative technique (Read et al., 2000; Niesters, 2002; Klein, 2002; Mackay, 2004; Iijima et al., 2004; Espy et al., 2006; Schuurman et al., 2007a). In the field of parasitology, several real-time PCRs for the detection of intestinal parasites have been developed showing excellent specificity and sensitivity; they have been accepted as objective tools for case confirmation and as gold standard in the development of new diagnostic tools. Implementation of molecular methods in routine diagnostics made a major step forward with the development of the first multiplex real-time PCR for the simultaneous detection of the three most important diarrhoea-causing protozoa (Verweij, 2004). Recent developments in improved and simplified DNA isolation and automation of these methods and the broader availability of real-time PCR-technology in diagnostic microbiology laboratories will allow these methods to be used as an alternative tool for the routine diagnosis of intestinal parasitic infections (Verweij, 2004; Espy et al., 2006). In microbiology laboratories the high specificity of DNA-based methods is one of major advantages and at the same time one of the major constraints as these methods will only detect the specific DNA targets for which primers and detection probes have been designed. This is in contrast with the “overall view” allowed by conventional diagnosis of intestinal parasites using microscopy. This thesis describes a number of studies on the implementation of real-time PCR for the detection and quantification of intestinal parasites to validate and optimize its use in different diagnostic settings and in epidemiological surveys.

SAMPLE COLLECTION

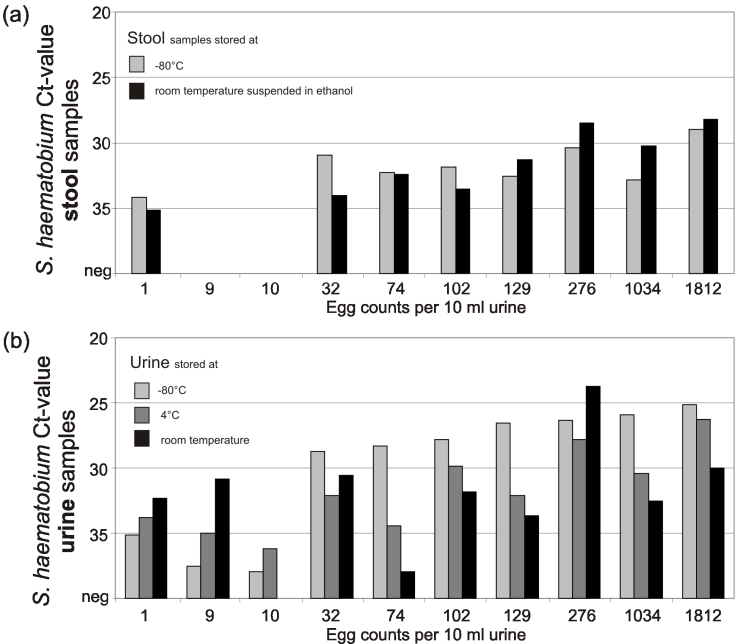
In industrialized countries it is common practice that stool samples are sent to the laboratory by regular mail. This delay between stool production and analysis has little or no effect on the microscopic detection of parasite eggs and cysts. The same was shown to apply for the detection of parasitic DNA with real-time PCR: in a study on the DNA detection of *Dientamoeba fragilis*, a parasite which lacks a cyst stage, isolation and the outcome of the PCR was reproducible even after 8 weeks of storage of faecal samples at 4°C (Verweij et al., 2007b).

For epidemiological studies, the storage of stool samples might nevertheless be a major problem. It is not exceptional that these studies are conducted in remote areas with a (sub)tropical climate, where power cuts are a daily phenomenon. To avoid degradation of collected samples, faecal specimens can be suspended in ethanol which will preserve the samples for at least three months even at tropical temperatures. This is illustrated by the detection of *Schistosoma haematobium* DNA in stool samples collected from 10 infected persons (figure 11.1). Although

S. haematobium eggs are normally excreted via the urine, in **chapter 9** it was shown that *S. haematobium* DNA detection in stool is feasible. Aliquots of stool samples were stored either frozen or were suspended in ethanol and stored at ambient temperatures. Figure 11.1a shows that the amplifications of isolated *S. haematobium* DNA were similar between the different storage methods. Storage and transport of non-fixated stool samples at ambient temperature for control was not feasible under tropical conditions. For urine, *S. haematobium* DNA was detected in most samples stored at three different temperatures (figure 11.1b). However, the temperature at which the urine samples were stored does influence the quantitative results.

Chapter 10 describes a sensitive method for the detection of *S. haematobium* DNA from vaginal lavages. These samples were collected under difficult conditions and at that time the researchers postulated that a more simple collection procedure and diagnostics was necessary. The use of vaginal swabs already proved to be a simple and sensitive method for the detection of several female genital infections (Whiley et al., 2005; Caliendo et al., 2005; Jatou et al., 2006). It is therefore worthwhile to investigate whether vaginal swabs could also be applied for the diagnosis of female genital schistosomiasis. For epidemiological studies, reliable storage methods which preferably allow the intensity of infection to be (semi-)quantified, still need to be tested both for urine samples and vaginal swabs.

Figure 11.1. Detected *Schistosoma haematobium* Cycle threshold (Ct)-values of DNA isolated from stool samples (a) collected from 10 patients living in an endemic area with *S. haematobium* infection determined by microscopic egg counts in urine. Samples of stool were stored in frozen condition or as ethanol suspension at room temperature over a period of two weeks. Additionally, urine samples (b) were collected and divided in three aliquots, with each aliquot stored at a different temperature for a period of two weeks. No ethanol or other fixatives were added to the urine. *S. haematobium* Ct-values were detected from DNA isolated from the urine samples. Urine and stool samples were collected and kindly provided by Dr. Akim Adegnika in collaboration with the medical research unit of the Albert Schweitzer Hospital in Lambaréné, Gabon.



SAMPLE ANALYSIS

The isolation of parasitic DNA is one of the most important steps for the implementation of DNA-based methods. It is therefore essential to check for each new target whether the isolation procedure is capable of extracting the DNA from the faecal sample. For example, a *Trichuris trichiura*-specific PCR was successful for the detection of *T. trichiura*-DNA extracted from an adult worm, however, isolation of DNA from the *T. trichiura* eggs in faeces failed. Several rough treatments (e.g. sonification and microwaving) have been used in vain to release the DNA from the eggs. DNA isolation from *T. trichiura* eggs remains a challenge, even more so when considering that the method should be feasible for routine sample preparation.

Faecal contaminants can cause inhibition of the DNA amplification. An internal control is therefore essential to monitor the efficiency of the amplification process. One of the targets of a multiplex assay amplifies and detects Phocin Herpes virus (PhHV) (Niesters, 2002), of which a fixed amount is added to one of the isolation buffers. A higher Ct-value than expected or the absence of the signal indicates a less efficient amplification process which can result in a false negative outcome of one of the other targets. PhHV is preferred because of simplicity and standardization: the same buffer with the internal control is also used for all multiplex assays and for other samples, such as tissue or urine samples. Detection of PhHV amplification does not necessarily mean that faecal DNA has been isolated. DNA amplification from normal flora found in the gastro-intestinal track has been suggested as control for faecal DNA isolation. However, the amount of normal gut flora fluctuates between samples resulting in different Ct-values and as a consequence (partial) inhibition would not be recognized. Another suggestion is to include one positive faecal sample for each target within a series of samples prepared for DNA isolation. This procedure would need sufficient supply of positive material and even then it still can not assure that DNA isolation in other samples has been successful.

Real-time PCR offers the ability to amplify and detect multiple DNA targets in a single reaction. Important advantages of a multiplex assay include reduced reagent costs and a high throughput potential. However, during the DNA amplification, inhibition can occur due to the simultaneous amplification of multiple targets in one assay. Part of designing a multiplex real-time PCR involves a background test, in which the amplification of one target is measured in the presence and absence of a second target. The background test shows the performance of the assay to detect a small number of DNA copies of one target in the presence of more abundant copy numbers of DNA from another parasite species. Combination of the real-time PCR for the detection of *Isospora belli* detection (Ten Hove et al., 2008) with the microsporidia real-time PCR (Verweij et al., 2007c) into one assay failed at this step. Amplification of multiple targets was not possible without the inhibitive effect on the

amplification of the microsporidia DNA. It is not clear yet whether the performance of this multiplex assay will improve by using another type of real-time PCR apparatus. Otherwise, a new design of these assays is needed.

Several real-time PCRs are already effectively employed in routine diagnosis and epidemiology. Because of the high specificity of real-time PCR, an assay could fail to detect genetically related species or strains (e.g. *Cryptosporidium* species and *E. histolytica* strains) due to variations in the DNA sequence of the primer and probe regions. Also, rare parasite species will be missed with standard real-time PCR panels. In patients with continuing gastro-intestinal complaints in which the initial real-time PCR screening does not reveal the cause of the complaints, microscopy is still required as an additional tool. The challenge for the future will be to maintain a profound knowledge on the morphological characteristics of these more difficult, “academic” cases.

In routine diagnostics and in epidemiology, often large numbers of samples are processed. With increasing numbers of samples, the amount of hands-on time for sample preparation and analysis can become a major bottleneck and human errors might occur more easily. DNA isolation and real-time PCR can be incorporated into a high throughput robotic workstation for an automated diagnostic process and eliminate these difficulties. Already several automated nucleic acid extraction platforms are available for various specimens and applications (Jongerijs et al., 2000; Kessler et al., 2001; Espy et al., 2001; Knepp et al., 2003). Beside the reduction in hands-on time, improved consistency of processes and reproducibility of DNA-extraction and -analysis will improve standard operation procedures in the laboratory.

The following sections will further discuss the diagnostic and epidemiological aspects of specific parasite species.

GIARDIA LAMBLIA

Giardia lamblia is worldwide one of the most common intestinal parasites causing gastro-intestinal complaints. Real-time PCR for this parasite is therefore considered as the first choice as a target in a diagnostic panel. Technical evaluation of the PCR has already demonstrated 100% specificity and a higher sensitivity as compared to microscopy and antigen tests (Verweij et al., 2003b, 2004a). Table 11.1 gives the results of the diagnostic tests for *G. lamblia* in a routine academic setting (Clinical Laboratory for Microbiology, Leiden University Medical Centre). It is clear that real-time PCR shows the highest sensitivity compared to the other tests for the detection of *G. lamblia*. In this thesis, *G. lamblia* real-time PCR was evaluated as part of a routine diagnosis for intestinal parasites in laboratories in Belgium and in The Netherlands. In previous studies, *G. lamblia* has been reported to be a common

pathogen in The Netherlands with a prevalence of 5% among general practice patients with gastro-intestinal complaints (De Wit et al., 2001c). A comparable prevalence is reported in **chapter 2** after routine screening with microscopy of general practice patients. When real-time PCR was applied on the same samples, the prevalence increased to 9.3%. In cases for which no microscopical examination for intestinal parasites had been requested by the general practitioners, *G. lamblia* was detected in 6.5% of the samples. This finding concurs with those from two other studies (Mank et al., 1995a; Van den Brandhof et al., 2006). Mank et al. reported that more than a third of detected pathogenic protozoa, mainly *G. lamblia*, would have been missed if the laboratory would have complied with the general practitioners' requests and Van den Brandhof et al. concluded that a test request by general practitioners did not always comply with existing knowledge of the etiology of gastro-enteritis. Another reason for missed infections mentioned in the latter study is the general restrictive policy in The Netherlands with regard to requesting tests.

Table 11.1. Test results (- = negative and + = positive) retrieved from the validation report for implementation of real-time PCR in routine diagnosis of *Giardia lamblia* at the clinical laboratory for microbiology at Leiden University Medical Centre (Unpublished data from Dr. J.J. Verweij).

Microscopy	Antigentest ^a	Real-time PCR	Number of samples	Ct range	Median Ct
-	-	-	254		
+	+	+	75	20,1 – 38,0	26,1
-	+	+	9	27,3 – 36,7	32,0
-	-	+	24 ^b	30,7 – 42,8	35,8
			362		

^a Alexon-Trend ProSpecT EIA for the detection of *G. lamblia*.

^b These samples include: 10 samples from four patients where in successive samples *G. lamblia* was also detected by microscopy and/or antigen test; 11 samples from six patients where *G. lamblia* infection was found in a family member; 3 samples from three patients in which only one out of a series of three was positive with PCR.

Although *G. lamblia* is regarded as a pathogenic parasite, in case-control studies it was also found in controls (Whitty et al., 2000; De Wit et al., 2001c; Hörman et al., 2004; Amar et al., 2007). In **chapter 4**, half of all detected cases of *G. lamblia* originated from returned travellers without gastro-intestinal complaints. The *G. lamblia*-positive samples were further characterized in **chapter 5** to analyse the role of *G. lamblia* assemblages (i.e. defined group of genotypes) in symptomatic and asymptomatic returned travellers. The data showed that increasing *G. lamblia* Ct-values are in concordance with decreasing gastro-intestinal complaints, translated as symptoms-score. However, the assemblages of *G. lamblia* revealed no association

with the presence or absence of any clinical symptoms in this group of travellers. Previous studies have suggested the epidemiological role of *G. lamblia* assemblages in children as an important factor associated with gastro-intestinal symptoms (Read et al., 2002; Haque et al., 2005; Sahagun et al., 2008; Kohli et al., 2008; Pérez Cordón et al., 2008). In adults, like most subjects in **chapter 4** and **5**, the association of assemblages with gastro-intestinal symptoms is less evident and can even give contradictory results (Homan & Mank, 2001; Gelanew et al., 2007; Sahagun et al., 2008). It has been suggested that the presence of symptoms in adults is more likely to be related with underlying conditions such as the degree of host adaptation, nutritional and immunological status. The results of studies on the association of *G. lamblia* assemblages with gastro-intestinal complaints are summarized in table 11.2.

Table 11.2. Summary of studies on the association of *Giardia lamblia* assemblages with clinical symptoms.

Reference	Study area	Study group	Gene target	Clinical symptoms	
				Assemblage A	Assemblage B
Homan et al., 2001	Netherlands	n=18; 8-60yrs	GDH	Mild, intermittent diarrhoea	Severe, persistent diarrhoea
Read et al., 2002	Australia	n=23; <5yrs	SSU-rDNA	Symptomatic	Asymptomatic
Haque et al., 2005	Bangladesh	n=211; <10yrs	SSU-rDNA	Symptomatic	Asymptomatic
Gelanew et al., 2007	Ethiopia	n=80; all ages	β-giardin	Less symptomatic	More symptomatic
Sahagun et al., 2008	Spain	n=72; <5yrs	TPI	More symptomatic	Less symptomatic
		n=65; 5-72yrs	TPI		No difference
Peréz et al., 2008	Peru	n=201; 0-9yrs	GDH	Diarrhoeic	High shedding, non diarrhoeic
Kohli et al., 2008	Brazil	n=189; children	SSU-rRNA		No difference
Ten Hove et al., 2008	Worldwide	N=156; mostly adults	TPI		No difference

Future studies on transmission patterns in households in combinations with detailed description of underlying conditions might provide more insight on the role of *G. lamblia* assemblages on the clinical presentation.

CRYPTOSPORIDIUM

Cryptosporidium infections were studied in both general practice patients and returned travellers. Microscopic detection of *Cryptosporidium* requires additional staining methods to visualize the parasite in faecal samples. This additional diagnostic procedure is not always included in routine stool analysis or requested by the health care providers, probably because cryptosporidiosis is usually associated with immunodepression in the context of HIV/AIDS. Given the sporadic requests

from the health care providers for additional *Cryptosporidium* detection, prevalence of cryptosporidiosis is highly underestimated (**chapter 2**) (Van Gool et al., 2003; Jones et al., 2004). The prevalence of *Cryptosporidium* in patients with diarrhoea as observed in **chapters 2** and **3** has so far exceeded the figures presented in other studies (Petry, 1998; Banffer & Duifhuis, 1989; Rodriguez-Hernandez et al., 1996; De Wit et al., 2001a; Vandenberg et al., 2006a). In the month of September 2005, *Cryptosporidium* (mainly *C. hominis*) was detected in up to 30% of all children aged under five with gastro-intestinal complaints. The reason for the high prevalence described in these chapters could be due to the unselected screening of patients by highly sensitive real-time PCR analysis in combination with sample collection during a seasonal peak of cryptosporidiosis (Van Asperen et al., 1996; Wielinga et al., 2007). A common source of infection has not been identified but cases did not show any geographic clustering (unpublished observations). A low prevalence of *Cryptosporidium* was shown for the group of mainly adult travellers, even among those who returned from 'high risk areas'.

ENTAMOEBA HISTOLYTICA

The very low prevalence of *Entamoeba histolytica* infections in the general practice patient population of **chapter 2** could suggest that the *E. histolytica* assay is redundant in the diagnostic real-time PCR panel. Although infection with *E. histolytica* is rare in The Netherlands, the assay was included because of the major clinical importance for the patient and its potential to spread among household members (Stanley, 2003). Even though a clinical *E. histolytica* infection is normally associated with tropical areas, this is not always evident and can therefore delay the diagnosis (Vreden et al., 2000; Edeling et al., 2004; Veneman et al., 2006). Such a case emerged during screening of an additional 1000 general practice patients as described in **chapter 3** for *Cryptosporidium* with the *E. histolytica*-*G. lamblia*-*Cryptosporidium* PCR (HGC-PCR). The *E. histolytica* real-time PCR positive individual concerned a young male with severe diarrhoea and an unknown travel history. Despite several microscopic analyses over a period of more than 3 years, still no pathogen was detected that could explain the gastro-intestinal complaints. Eventually the patient was treated as a clinical amoebic infection (T. Schuurman, personal communication). As described in the introduction, the classical method for detection and diagnosis of *E. histolytica* infection is not that straightforward and it is essential that physicians are familiar with epidemiology, clinical syndromes, management of infections and available diagnostic tests. The introduction of the HGC-PCR, in particular for travellers, greatly facilitates the difficult diagnosis of *E. histolytica* infection.

STRONGYLOIDES STERCORALIS

The diagnosis of a *Strongyloides stercoralis* infection by microscopy involves multiple

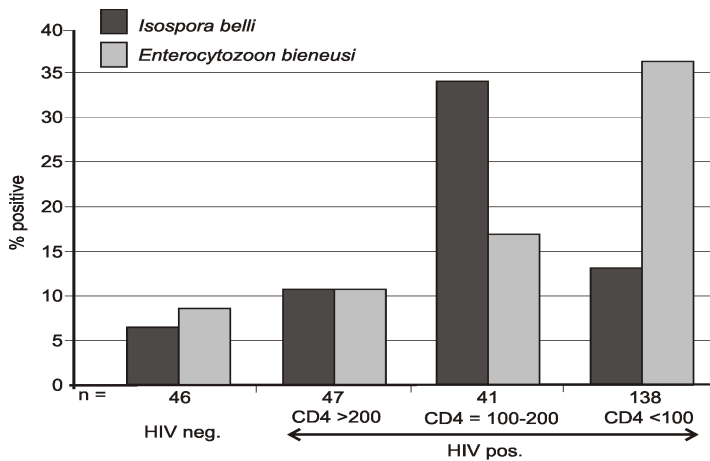
sampling and specific concentration methods. The Baermann concentration method is widely used, but the sensitivity of the test depends on the number of samples examined. Furthermore, there are several reasons why this *S. stercoralis* detection procedure is omitted, like in **chapter 4** because the samples were too old, did not have the right consistency, or only a small amount of faeces was available. Moreover, it was shown that physicians often do not consider strongyloidiasis in their differential diagnosis, which could place patients at iatrogenic risk (Loutfy et al., 2002; Boulware et al., 2007). In the setting described in **chapter 4**, the screening for *S. stercoralis* infection with real-time PCR proved rewarding, but may also be an important method to avoid hyper-infection in patients in need of immunosuppressive treatment.

ISOSPORA BELLI AND MICROSPORIDIA

In the epidemiology of opportunistic intestinal parasitic infections in immunosuppressed and immunoincompetent patients, real-time PCR proved to be a useful tool for the detection of *Isospora belli* (**chapter 6**) and microsporidia (**chapter 7**) infections. In Blantyre, Malawi, the real-time PCRs were used for a study on the aetiology of diarrhoea in hospitalized patients (figure 11.2). Stool samples were collected from HIV/AIDS-positive ($n = 226$) and HIV-negative ($n = 46$) patients. *I. belli* and *Enterocytozoon bieneusi* were detected by real-time PCR in 175 (64%) and 197 (73%) of all cases, respectively. Preliminary data analyses show that the intensities of the parasitic infections (reflected in Ct-values) were associated with the severity of diarrhoea (Beadsworth, unpublished data). Moreover, *I. belli* was more common in patients with 100-200 CD4-cells/mm³, while *E. bieneusi* was mostly detected in patients with <100 CD4 cells/mm³ (figure 11.2).

The decrease in the number of *I. belli* infections in the last patient group is not necessarily the result of the lower CD4 counts (Kartalija & Sande, 1999; Certad et al., 2003). It could also be due to the in vivo competition between the two parasitic infections. Further data analysis, including the results of *Cryptosporidium* detection is still pending (Beadsworth et al., in preparation).

Figure 11.2. Percentage of detected *Isospora belli* positive cases and *Enterocytozoon bieneusi* cases with detected Ct-values below 35, in HIV-negative (n = 46) and -positive (n = 226) patients categorized by their HIV status (reflected by CD4 counts). Samples were collected from patients in Blantyre, Malawi (Unpublished data from L. van Lieshout, M.B. Beadsworth).



Because of higher awareness of microsporidiosis together with the availability of the highly sensitive and specific diagnostic techniques based on PCR, microsporidia infections are increasingly diagnosed in transplant recipients, children, seniors and travellers. The detection of microsporidia infections in these patient populations in western settings is mostly clear cut. However, in the study in Malawi, but also in unpublished observations in Ethiopia, a very high prevalence of *E. bieneusi* infections was observed. In half of the detected cases Ct-values were above 35 reflecting a very low DNA-load. The clinical relevance of these findings is not clear. Possible explanations for these cases could be just an infection with a low parasite load, the presence of asymptomatic carriers or the results of an ubiquity of spores in tropical environments with high HIV/AIDS prevalence among the population (Van Gool et al., 1997; Cegielski et al., 1999; Tumwine et al., 2002; Siński, 2003; Nkinin et al., 2007; Samie et al., 2007; Raccurt et al., 2008). To elucidate the association of microsporidia infections with different clinical backgrounds, *E. bieneusi* genotypes were characterised in several patient cohorts. The phylogenetic study in **chapter 8** describes a clustering in different human populations for some of the genotypes of *E. bieneusi*. Using the same techniques for detection and characterization, future studies could attempt to follow transmission patterns involving (asymptomatic) household members, domestic animals and the environment to determine the potential of *E. bieneusi* as a zoonotic and water-borne pathogen.

SCHISTOSOMA SPP.

In **chapter 9** a multiplex real-time PCR approach on the cytochrome C oxidase subunit 1 (*cox1*) gene was compared with egg counts determined by Kato-smears on preselected series of faecal samples. These were collected from subjects living in an area endemic for both *Schistosoma mansoni* and *Schistosoma haematobium*. The multiplex real-time PCR showed 100% specificity based on an extensive number and variety of controls and 100% sensitivity in subjects with more than 100 *S. mansoni* eggs per gram faeces, with few false negatives in individuals excreting lower numbers of eggs. Although eggs of *S. haematobium* were not observed in stool samples, schistosome DNA could successfully be detected. As expected, the sensitivity of *S. haematobium* DNA detection in faeces is lower than the sensitivity achieved by microscopic examination of urine samples. Preliminary results using real-time PCR on DNA isolated from urine samples, collected in a *S. haematobium*-endemic area in Gabon showed promising results (figure 11.1). For further improvement on the sensitivity of the molecular test, another real-time PCR was developed targeting the internal transcribed spacer 2 (ITS-2) (Obeng et al., 2008). The ITS-2 PCR improved *Schistosoma* detection considerably, in particular for the detection of *S. mansoni* (table 11.3). Unlike the *cox1* gene, the real-time PCR assay on ITS-2 does not discriminate between DNA of *S. mansoni* and DNA of *S. haematobium*.

Table 11.3. Amplification of a specified amount of *Schistosoma mansoni* DNA and *S. haematobium* DNA isolated from adult worms, with the species specific *cox1* real-time PCR assay and the genus specific ITS-2 real-time PCR assay reported in detected Ct-values.

Target DNA	Schistosoma mansoni		Schistosoma haematobium	
	<i>cox1</i> PCR	ITS-2 PCR	<i>cox1</i> PCR	ITS-2 PCR
100 ng	18,0	14,8	17,2	16,3
10 ng	21,8	17,2	20,5	19,5
1 ng	25,9	20,9	24,1	23,1
100 pg	29,9	24,4	27,7	26,7
10 pg	33,6	27,9	31,3	29,0
1 pg	37,6	31,2	33,8	34,4
100 fg	41,9	36,0	39,0	38,5
10 fg	0	0	0	0

The ITS-2 PCR was further evaluated using urine samples collected in Ghana and compared with circulating cathodic antigen (CCA) strips and microscopy (Obeng et

al., 2008). The results indicate that real-time PCR may potentially serve as a gold standard to determine the prevalence and intensity of *Schistosoma* infections in surveys. Moreover, these results and the findings described in **chapter 9** suggest the versatility of real-time PCR as a diagnostic tool for urine, stool and other clinical samples. In **chapter 10** the performance of the *Schistosoma* real-time PCR was evaluated for the diagnosis of female genital schistosomiasis on DNA isolated from vaginal lavages. This preliminary investigation showed that *Schistosoma* real-time PCR is a valuable tool for diagnosis in epidemiology.

MULTIPLEX REAL-TIME PCR FOR ROUTINE DIAGNOSTICS

The microscopic detection of parasites is generally accepted as the gold standard, although it has also been widely acknowledged that it falls short on effectiveness in both patient care and epidemiology.

Each year approximately 320.000 Dutch patients with gastro-intestinal complaints consult their general practitioner (Van Duynhoven et al., 2005). Guidelines for general practitioners to request stools were created by the Dutch Society of General practitioners (NHG) (Brühl et al., 2007) to improve daily practice for managing patients with gastro-intestinal complaints and can be summarized as follows:

The guidelines for the diagnostic standard for acute diarrhoea recommends additional diagnostic analysis for intestinal protozoa when the diarrhoea continues for more than 10 days. The GP should ask for a fresh stool sample for it to be analysed within hours after production. When the outcome is negative, two more stool samples should be requested, produced with an interval of a few days. If available, preference should be given to the TFT procedure. If no intestinal parasites were discovered in three samples and protozoan infection is still suspected, the laboratory could be consulted to perform a copro-antigen test for *G. lamblia* detection. Detection of *E. histolytica* with PCR and *Cryptosporidium* with specific staining methods have to be explicitly asked for as these techniques are usually not performed on a routine basis. (Translated from Dutch by R. ten Hove).

Although the diagnostic standard procedure for general practitioners as described above is fairly straightforward, many additional patients visiting their general practitioner were diagnosed with *G. lamblia*- and *Cryptosporidium*-infection by the molecular diagnostic approach (**chapter 2** and **chapter 3**). Detection of these cases with the conventional diagnostic approach had failed because cases did not meet the criteria for parasite examination or, even if cases answered the criteria for parasite examination, specific parasite infections were not suspected by the general practitioners. Last but not least, supported by the semi-quantitative results, real-time PCR has the ability to detect very low loads of intestinal parasites that would most likely be missed with microscopic and antigen detection methods.

The design of real-time PCR panels for specific patient groups could improve the standard diagnostic procedures and patient diagnostics (Verweij, 2004). The advantages of such real-time PCR panels can further be supported by the results of **chapters 2, 3 and 4**. Table 11.4 gives examples of real-time PCR panels that could be used in different patient groups. In children, *G. lamblia* and *Cryptosporidium* are two important pathogens. Although *D. fragilis* has been suggested to be also a potential pathogen in children, more studies are needed to support this statement. Some of the microbiological diagnostic laboratories in The Netherlands have replaced microscopic examination of SAF-preserved stool samples with a real-time PCR panel including a *D. fragilis*-specific PCR which can help to elucidate the pathogenic role of this parasite (Bruijnesteijn van Coppenraet et al., n.d.).

Table 11.4. Real-time PCR assays that were developed and evaluated summarized as components of different patient groups. Several targets have also been combined and evaluated as multiplex real-time PCR assays.

Adults	Children
<i>Entamoeba histolytica</i> <i>Giardia lamblia</i> <i>Cryptosporidium</i>	<i>Dientamoeba fragilis</i> <i>Giardia lamblia</i> <i>Cryptosporidium</i>
Travellers	Immune compromised
<i>Entamoeba histolytica</i> <i>Giardia lamblia</i> <i>Cryptosporidium</i> <i>Strongyloides stercoralis</i> <i>Schistosoma</i> ^b <i>Cyclospora cayetanensis</i> ^b <i>Isospora belli</i> ^b <i>E. bienersi</i> ^c	<i>Strongyloides stercoralis</i> ^a <i>Cryptosporidium</i> <i>Isospora belli</i> <i>Enterocytozoon bienersi</i> <i>Encephalitozoon</i> spp.
a: Recommended when corticosteroid treatment is scheduled. b: Optional for those returning from high-risk areas. c: Actual prevalence among travellers needs further investigation.	

In immunocompromised patients the most important opportunistic intestinal parasites can be related to the conditions of the immune suppression. For example, *S. stercoralis* hyperinfection is associated with corticosteroid treatment and HTLV infection but not with HIV infection. Travellers comprise a very diverse group, including immigrants, adoption children, tourists, businessmen, expatriates, army personnel, etc. who may have been exposed to a wide spectrum of infectious micro-organisms. Many studies have assessed the relative frequency of intestinal parasitic diseases to characterize demographic and travel-related predictors of infections (Bottieau et al., 2006, 2007; Van De Winkel et al., 2007; Cobelens et al., 1998; Muennig et al., 1999; Von Sonnenburg et al., 2000; Whitty et al., 2000; Saiman et al.,

2001; Okhuysen, 2001, 2007; Ansart et al., 2005; Steffen, 2005; Bailey et al., 2006; Thors et al., 2006; Freedman et al., 2006; Seybolt et al., 2006; Caruana et al., 2006; Wilson et al., 2007; Sudarshi et al., 2003; Boggild et al., 2006; Brouwer et al., 1999). To reach a consensus on which diagnostic procedures to perform for this patient group remains very complicated. In **chapter 4** the difference in the number of detected cases using conventional- and the molecular-diagnostic approach was small. However, in the setting of a travel clinic the full arsenal of diagnostic tests are used to cover most of the intestinal pathogenic parasite species. Profit can be gained by simplifying these diagnostic procedures. An extensively automated diagnostic screening system for the most important parasites can be more cost-efficient. Nevertheless, it is still difficult to decide which parasite targets would be needed in a standard screening panel. Real-time PCR showed to be more effective in detecting all four targeted parasite species than conventional diagnostic methods. Moreover, only few additional parasite species were diagnosed with microscopy, while probably many more unexplained causes of gastro-intestinal complaints might be resolved by the application of additional real-time PCR targets.

CONCLUDING REMARKS

Real-time PCR has opened new perspectives for diagnosis of intestinal parasites. Nevertheless, the process of improving diagnostic methods is still continuing. It is evident that in the future the throughput of real-time PCR platforms will improve substantially by increasing the number of targets, speeding up sample processing time and introducing plates with a larger number of wells. Already a real-time PCR system has been assessed for the detection of five viral targets on 384 well format (Molenkamp et al., 2007). Moreover, diagnostic technologies, such as biosensors, micro-DNA arrays and microfluid technologies are advancing (Wang et al., 2004; Van Merkerk, 2005; Taguchi et al., 2005; Stein et al., 2006). At the same time, new parasitic diseases can emerge while others disappear. Therefore, any diagnostic method, new or old, should only be applied when it has proven to provide an added diagnostic value. The present thesis shows that real-time PCR provides a feasible and effective alternative method for the routine diagnosis of intestinal parasites in patient care and epidemiology.

The initial implementation of real-time PCR in a laboratory requires a large investment. On the other hand prices of the equipment and consumables continue to decrease and become more competitive compared with the high costs of staff in a laboratory. Cost-effectiveness of real-time PCR could increase when diagnostic assays are extended with additional parasitic, bacterial, fungal and/or viral targets. Nevertheless, it needs to be taken into consideration that although cost efficiency regarding consumables and labour-time might favour one diagnostic methodology, the amount of reimbursement for a laboratory can still vary extensively due to

different declaration procedures in various health care systems.

A major challenge that remains to be solved is the access to sensitive and specific diagnostic tools in low income countries. Although the real-time PCR method brings forward new exciting prospects for epidemiology of intestinal parasites in remote and endemic areas, it has little value for clinical diagnostics in most of these settings. However, real-time PCR can play a prominent role as a gold standard in quality control for the evaluation of new inexpensive rapid tests for field diagnostics. Ultimately, field-applicable PCR formats can be developed with instruments being further miniaturized with integrated sample processing (Gerdes et al., 2008; Yager et al., 2008). Such advances would have a significant impact on public health in resource-poor settings.

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SUMMARY

The detection of intestinal parasitic infections for routine diagnosis and for epidemiological research still depends mainly on microscopical examination of stool samples for the identification of helminth eggs and protozoan trophozoites and cysts. Because microscopy has several limitations, additional diagnostic methods (e.g. culture, antigen detection) have been introduced to improve the detection of intestinal parasites. Although such additional methods increase sensitivity, the amount of hands-on time accumulates substantially. In **chapter 1** various diagnostic methods are summarized and the diagnostic challenges are described for the most important intestinal parasitic infections.

During the last years remarkable progress has been made in the development of molecular diagnostic methods based on the Polymerase Chain Reaction (PCR) technique. For the detection of intestinal parasites, DNA isolation from stool can be processed in a semi- or fully-automated system where after specific DNA of multiple targets can be amplified simultaneously, visualized on screen and semi-quantified in a closed tube system with a multiplex real-time PCR. In this thesis, a diagnostic approach using multiplex real-time PCR is assessed for the routine clinical diagnosis and epidemiology of intestinal parasites.

In **chapter 2** the diagnostic results that were obtained with multiplex-real-time PCR for the detection of *Entamoeba histolytica*, *Giardia lamblia* and *Cryptosporidium parvum* / *C. hominis* (HGC PCR) were compared with the results obtained by routine microscopy on faecal samples from patients visiting their general practitioner because of gastrointestinal symptoms. The study revealed that significant numbers of *G. lamblia* and *Cryptosporidium* infections remained undetected with microscopy, even with the use of a multiple sampling procedure of faecal specimens in combination with fixatives (also referred to as the triple faeces test (TFT) procedure). Parasites that had been detected with microscopy but not with real-time PCR consisted mainly of non-pathogenic parasites and *Dientamoeba fragilis*, of which its pathogenicity is still disputed upon. Hence, compared to the molecular approach, microscopy provided limited sensitivity in a routine diagnostic setting of general practice patients with gastro-intestinal complaints.

For the same patient group as described in **chapter 2**, the results of *Cryptosporidium* detection with the HGC-PCR were studied in more detail in **chapter 3**. Microscopic examination for *Cryptosporidium*, which requires an additional staining procedure of the faecal smear, was specifically requested by general practitioners twenty-one times over a period of approximately seven months and was found positive in 13

cases. In contrast to the conventional diagnostic approach, with HGC PCR 80 cases were detected. The prevalence of *Cryptosporidium* in Dutch patients with gastro-intestinal complaints attending their general practitioner has so far exceeded the figures in previous studies. The highest infection rate has been detected among children aged under five years with a peak in the month of September: almost one-third of them had been infected with *Cryptosporidium*, mainly with the species *Cryptosporidium hominis*. The lack of request for an additional diagnostic procedure to detect *Cryptosporidium* is not the only reason for missing infections. Basic microscopic stool examination was often not requested, leaving a substantial number of gastro-intestinal parasitic infections undiagnosed.

Besides the Dutch general practice patients group, the diagnostic approach with real-time PCR was also assessed for the routine diagnosis of intestinal parasites in returning travellers. **Chapter 4** describes a prospective study where the results of 2591 microscopic examinations and antigen detections (i.e. care as usual) were compared with the results of those from the HGC-PCR and an additional *Strongyloides stercoralis* real-time PCR. The detection rates of all four targeted parasite species were increased with real-time PCR whereas the prevalence of ten additional pathogenic parasite species found with microscopy was 0.5% at most. A fully automated DNA isolation process and extension of the molecular diagnostic targets could further increase the detection rates of parasites while having considerable impact on cost-efficiency of the diagnostic procedures in the laboratory.

Chapter 5 describes the molecular epidemiology of *G. lamblia* infections in the group of returned travellers. Symptoms of *G. lamblia* infections in this group, consisting mainly of adults, were highly variable and ranged from asymptomatic to the presence of severe gastro-intestinal complaints. Data in this study showed that although the *G. lamblia* Cycle-threshold (Ct)-values, which reflect the intensity of infection, correlated with the number of specified gastro-intestinal complaints, the parasite was also detected in 4.7 % of the travellers who did not have any intestinal symptoms. The reasons for the clinical heterogeneity of *G. lamblia* infections are still not fully understood. In previous studies the epidemiological role of *G. lamblia* assemblages (i.e. group of genotypes) have been suggested as an important factor associated with gastro-intestinal complaints, however, this could not be proven in this group of travellers.

In **chapter 6** a real-time PCR for the detection of *Isospora belli* was evaluated and in **chapter 7** a multiplex real-time PCR was evaluated for the detection of *Encephalitozoon intestinalis* and *Enterocytozoon bieneusi*. These real-time PCRs have been developed in addition to the panel of molecular diagnostic assays to be used in a clinical setting and in epidemiological studies.

As such, a phylogenetic study of *E. bieneusi* infections in persons with different

clinical backgrounds is described in **chapter 8**. The results indicate a dynamic evolutionary process between genotypes of *E. bieneusi*. One specific genotype was restricted to transplantation patients receiving immuno-suppressives and another genotype showed its preferential habitat in patients living with HIV/AIDS, which further emphasizes the predisposition for specific hosts by different *E. bieneusi* genotypes.

Although *Schistosoma mansoni* and *Schistosoma haematobium* are living in the blood vessels and not in the intestinal lumen, this thesis also elaborates on real-time PCR for the detection of *Schistosoma* in humans. In **chapter 9** a multiplex real-time PCR was evaluated for the detection of both *S. mansoni* and *S. haematobium* in stool samples collected in an area endemic for both *Schistosoma* species. The detected Ct-values correlated with the results from quantitative microscopy on stool and on urine samples. Furthermore, the use of ethanol-suspended stool samples for storage and transport at ambient temperatures, as was done in this study, makes collection of samples at remote areas more attractive. In **chapter 10**, the performance of the *Schistosoma* real-time PCR was evaluated for the diagnosis of female genital schistosomiasis on DNA isolated from vaginal lavages. The preliminary results in this study showed that the semi-quantitative outcome of the PCR can be used in the differential diagnosis of vaginal lesions and as a predictor of disease pathology.

In conclusion, the comparative studies in this thesis revealed that the introduction of real-time PCR for routine detection of diarrhoea causing protozoa and helminths will improve the diagnostic efficiency of laboratories dealing with faecal samples. Standard diagnostic procedures can further be improved by the design of real-time PCR panels for specific patient groups containing parasitic, bacterial, fungal and / or viral targets. Furthermore, with the simple sample collection procedure and the high throughput potential, the multiplex real-time PCR showed to be a powerful tool for epidemiological studies, even in remote areas. The real-time PCR method has little value for clinical diagnostics in low-income countries but can certainly play a prominent role as a gold standard in quality control for the evaluation of new, inexpensive rapid tests for field diagnostics.

SAMENVATTING

De detectie van parasitaire darminfecties binnen de routinematige diagnostiek en voor het epidemiologisch onderzoek is nog altijd in hoge mate afhankelijk van microscopisch onderzoek van de ontlasting. Door middel van microscopie van feces monsters kunnen wormeieren en protozoaire trofozoiten en cysten herkend worden. Aangezien microscopie een aantal beperkingen heeft, zijn additionele diagnostische methoden nodig (bijvoorbeeld larvenkweek en antigeneendetectie) voor het aantonen van darmparasieten. Hoewel deze aanvullende methoden de gevoeligheid van de diagnostiek aanzienlijk kunnen verbeteren, kost dergelijk onderzoek extra arbeidstijd.

Hoofdstuk 1 geeft een algemene introductie over de verschillende diagnostische methoden en de uitdagingen in de diagnostiek van de belangrijkste parasitaire darminfecties. In de laatste jaren is een grote vooruitgang geboekt in de ontwikkeling van moleculaire diagnostische methoden, die zijn gebaseerd op de Polymerase Chain Reaction (PCR) techniek. DNA isolatie uit de ontlasting kan worden gerealiseerd in een semi- of volledig geautomatiseerd systeem. Hierna kan het specifieke DNA van meerdere ziekteverwekkers gelijktijdig kunnen worden vermeerderd en semi-kwantitatief worden aangetoond in een gesloten systeem met behulp van 'multiplex real-time PCR'. Eerder onderzoek liet zien dat de multiplex real-time PCR een zeer gevoelige en specifieke methode is om darm-parasieten aan te tonen. In dit proefschrift worden verschillende studies beschreven waarin deze moleculair diagnostische aanpak verder werd uitgebreid en gevalideerd, zowel voor de toepassing in routinematig patiëntendiagnostiek als voor epidemiologisch onderzoek.

In **hoofdstuk 2** worden de resultaten gepresenteerd van een onderzoek waarin de *Entamoeba histolytica*, *Giardia lamblia* en *Cryptosporidium* multiplex real-time PCR (HGC PCR) is gebruikt voor het testen van feces-DNA-monsters afkomstig van 950 patiënten uit de regio Groningen die hun huisarts bezochten vanwege darmklachten. De resultaten van de HGC-PCR werden vergeleken met routinematig uitgevoerd bacteriologisch en parasitologisch onderzoek. Uit deze studie bleek dat grote aantallen van *G. lamblia* en *Cryptosporidium* infecties onopgemerkt bleven met microscopie, zelfs wanneer meerdere monsters van een patiënt werden onderzocht met de zogenaamde de 'triple feces test (TFT)-procedure. Parasieten die wel opgespoord werden met de microscopie, maar niet werden aangetoond met real-time PCR betroffen voornamelijk niet-pathogene parasieten en *Dientamoeba fragilis*, waarbij van deze laatstgenoemde de pathogeniteit nog niet geheel duidelijk is. Bovendien werden parasitaire infecties gemist omdat parasitologisch onderzoek

veelal niet werd aangevraagd door de huisarts.

In **hoofdstuk 3** wordt een aanvullend onderzoek beschreven dat zich specifiek richt op de epidemiologie van *Cryptosporidium* infecties in 1.914 patiënten met darmklachten. Microscopisch onderzoek voor *Cryptosporidium*, waarbij aanvullend onderzoek in de vorm van zuurvaste kleuring nodig is, bleek door de huisarts slechts 21 keer te zijn aangevraagd waarvan 13 gevallen positief, terwijl met de HGC-PCR 80 gevallen werden aangetoond. Het hoogste infectiepercentage werd aangetroffen bij kinderen jonger dan vijf jaar met een piek in de maand september. Bijna één-derde van de jonge kinderen was geïnfecteerd met *Cryptosporidium* ten tijde van deze piek voornamelijk met *Cryptosporidium hominis*.

Naast de Nederlandse patiëntengroep uit huisartspraktijken, werd de diagnostische aanpak op basis van real-time PCR ook geëvalueerd voor de routinematige diagnostiek van darmparasieten bij terugkerende reizigers. **Hoofdstuk 4** beschrijft een prospectieve studie waarin de resultaten van het microscopisch onderzoek en antigeen-detectie (de 'normale' procedure) van 2.591 feces monsters werden vergeleken met de resultaten van de HGC-PCR en een aanvullende *Strongyloides stercoralis* real-time PCR. Naast de verhoogde gevoeligheid van de toegepaste PCR's ten opzichte van de microscopie en antigeen testen, bedroeg de prevalentie van de overige microscopisch aangetoonde infecties maximaal 0,5% per parasietensoort. Door de sterk gereduceerde werklast zal een volledig geautomatiseerde DNA isolatieprocedure en de mogelijkheid tot uitbreiding van de DNA-targets een aanzienlijk effect hebben op de kostenefficiëntie van de diagnostische procedures in het laboratorium.

Hoofdstuk 5 beschrijft de moleculaire epidemiologie van *G. lamblia*-infecties in dezelfde groep reizigers. Symptomen van *G. lamblia*-infecties in deze groep, hoofdzakelijk bestaande uit volwassenen, waren zeer variabel en varieerden van asymptomatisch tot de aanwezigheid van ernstige darmklachten. Uit de resultaten van deze studie bleek dat de *G. lamblia* Cycle-threshold (Ct)-waarden, waarmee de intensiteit van de infectie wordt weerspiegeld, correleerden met het aantal specifieke darmklachten. Verder werd de parasiet geconstateerd bij 4,7% van de teruggekeerde reizigers die geen darmklachten hadden. De redenen voor de klinische heterogeniteit van *G. lamblia*-infecties zijn nog niet volledig duidelijk. In eerdere studies werd de epidemiologische rol van *G. lamblia*-assemblages (d.w.z. groep van genotypen) aangewezen als belangrijke factor in de ernst van de darmklachten. Een dergelijk verband kon niet worden aangetoond in deze groep van reizigers.

In **hoofdstuk 6** is een real-time PCR geëvalueerd voor de detectie van *Isospora belli* en in **hoofdstuk 7** is een multiplex real-time PCR geëvalueerd voor de detectie van *Encephalitozoon intestinalis* en *Enterocytozoon bieneusi*. Deze real-time PCRs zijn

ontwikkeld als uitbreiding op het panel van moleculair diagnostische tests in de klinische diagnostiek en epidemiologisch onderzoek. In **hoofdstuk 8** wordt een fylogenetische studie beschreven van *E. bieneusi*-infecties bij personen met verschillende klinische achtergronden. De resultaten van deze studie laten zien dat er een dynamisch evoluerend proces plaatsvindt tussen verschillende *E. bieneusi*-genotypen. In deze studie kon worden aangetoond dat bepaalde *E. bieneusi*-genotypen de voorkeur geven aan een specifiek soort gastheer. Genotype C beperkte zich bijvoorbeeld tot transplantatiepatiënten die immunosuppressieve medicatie krijgen toegediend terwijl andere genotypes voornamelijk werden gevonden bij HIV/AIDS patiënten.

Hoewel *Schistosoma mansoni* en *Schistosoma haematobium* parasieten zijn die leven in bloedvaten en niet in het darmlumen, wordt in dit proefschrift ook ingegaan op real-time PCR voor de detectie van *Schistosoma* bij de mens. In **hoofdstuk 9** wordt een multiplex real-time PCR geëvalueerd voor de detectie van zowel *S. mansoni* en *S. haematobium* DNA in feces monsters die verzameld zijn in een gebied dat endemisch is voor beide *Schistosoma* soorten. Voor het onderzoek met real-time PCR werden de feces-monsters gemengd met ethanol en konden daarom zonder koeling bewaard worden. De resultaten laten een correlatie zien tussen de gedetecteerde Ct-waarden van DNA uit feces en de resultaten van de kwantitatieve microscopie op feces en op urine monsters. Real-time PCR biedt daarom een goed alternatief voor onderzoek naar *Schistosoma*-infecties in endemische gebieden.

In **hoofdstuk 10** wordt beschreven hoe *Schistosoma* real-time PCR werd toegepast op DNA geïsoleerd uit vaginale spoelsels voor het aantonen van genitale schistosomiasis. Uit de resultaten van deze studie blijkt dat deze semikwantitatieve PCR kan worden gebruikt in de differentiaal diagnostiek van vaginale laesies.

De resultaten van de studies in dit proefschrift hebben duidelijk aangetoond dat de invoering van real-time PCR in routine diagnostische laboratoria vooruitgang zal bieden bij de patiëntendiagnostiek. Moleculaire diagnostische procedures zouden verder verbeterd kunnen worden door real-time PCR's voor parasieten, bacteriën, schimmels en / of virale targets te combineren voor specifieke patiëntengroepen. Steeds verdere vereenvoudiging en automatisering bij DNA isolatie en amplificatie maken het mogelijk om real-time PCR op grote schaal toe te passen in zowel de routine diagnostiek als epidemiologisch onderzoek. Real-time PCR kan in de klinische diagnostiek in ontwikkelingslanden een belangrijke rol spelen als gouden standaard bij de kwaliteitscontrole en evaluatie van nieuwe (goedkopere) snel-testen.

CURRICULUM VITAE

Robert-Jan ten Hove was born on the 31st of January, 1975 in Arnhem, The Netherlands. In 1992 he passed his HAVO school-exam and in 1994 his VWO school-exam at the Doorenweerth college in Doorwerth. From 1995 till 2000 he studied Biology at Wageningen University. His MSc. research projects were on Molecular Virology and Human Genetics. He did his practical at the department of Virology at the University of Cape Town in South-Africa. After graduation he worked at the department of Human Genetics at Leiden University Medical Centre, where he previously did his second research project. After half a year he decided to change direction and went to study for a post-graduate diploma in Tropical Medicine and Healthcare at the Prince Leopold Institute for Tropical Medicine in Antwerp, Belgium. In 2002 he started working for Doctors without Borders (MSF) for three years in various field projects. In between projects he worked for eight months at the Centre for Plant Disease of the Ministry of Agriculture, Nature and Food Safety in Wageningen. His last field-mission with MSF was a sleeping sickness eradication program in Congo-Brazzaville. In January 2005 he started his PhD research, as described in this thesis, at the department of Parasitology, Leiden University Medical Centre under the supervision of Prof. Dr. A.M. Deelder, Dr. E.A. van Lieshout and Dr. J.J. Verweij.

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