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Molecular detection of intestinal parasites for clinical diagnosis and epidemiology

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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-supressives 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.