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Molecular Testing for Clinical Diagnosis and Epidemiological Investigations of Intestinal Parasitic Infections

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SUMMARY

Over the past few decades, nucleic acid-based methods have been developed for the diagnosis of intestinal parasitic infections. Advantages of nucleic acid-based methods are numerous; typically, these include increased sensitivity and specificity and simpler standardization of diagnostic procedures. DNA samples can also be stored and used for genetic characterization and molecular

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typing, providing a valuable tool for surveys and surveillance studies. A variety of technologies have been applied, and some specific and general pitfalls and limitations have been identified. This review provides an overview of the multitude of methods that have been reported for the detection of intestinal parasites and offers some guidance in applying these methods in the clinical laboratory and in epidemiological studies.

INTRODUCTION

Soon after the first publications on the *in vitro* amplification of DNA by PCR (1, 2), it was predicted that this new technology would lead to a breakthrough in molecular parasitology and in the diagnosis of parasitic infections (3, 4). Until that time, specific DNA probes had been used in basic research and also in diagnostic applications despite being hampered by the limited sensitivity of such direct hybridization assays without a preceding amplification step. It was expected that this could be overcome by the specific amplification of minute amounts of DNA using PCR. In 1995, a large number of papers on DNA-based methods for the detection and identification of a range of parasitic infections were reviewed by J. B. Weiss (5). At that time, the use of PCR was still limited, and most research had been limited to malaria, *Leishmania*, trypanosome, and *Toxoplasma* parasites, all of which are tissue parasites. Moreover, except for toxoplasmosis, the experience of using PCR on DNA isolated directly from patient material was limited.

Over the last 10 to 15 years, many clinical microbiology laboratories have been provided with facilities for performing molecular diagnostics. Moreover, technical advances, especially the introduction of real-time PCR, have overcome many drawbacks of PCR from the early years, such as contamination risk from amplified products. It also became possible to combine more than one target in a multiplex assay relatively simply. In addition, the implementation of automated DNA/RNA isolation methods has made it possible to use nucleic acid-based detection techniques in a high-throughput format.

Molecular detection, differentiation, and genotyping methods for a large number of parasites have been described and implemented in both diagnostic and research settings. In this review, we focus primarily on molecular diagnostics and the molecular epidemiology of intestinal parasites and parasites that reside elsewhere in the body but whose DNA can be detected in fecal samples (e.g., *Schistosoma* and *Paragonimus*).

TECHNIQUES

PCR

First described in 1985, PCR allows the *in vitro* amplification of a specific DNA fragment in a cyclic process of denaturation, hybridization of primers, and elongation of the DNA strand using a thermostable DNA polymerase (1, 2). In nested PCR, amplicons from a PCR are used as the template in a second PCR using one primer ("seminested" or "heminested") or two primers different from those used in the initial PCR and located within the sequence amplified by the first primer set. Nested PCRs are used to increase sensitivity and specificity but have been more or less abandoned in diagnostic laboratories due to the risk of contamination by PCR products. The amplification products in a conventional PCR are usually visualized with ethidium bromide or alternative, less mutagenic dyes after agarose gel electrophoresis. Specificity is based on the expected size of the PCR product. Multiplex PCRs, combining PCRs for different DNA targets, can be achieved by choosing primers for each target in such a way that different-sized amplicons for each target are produced. Optimization of such conventional multiplex PCRs is difficult, as the efficiency of the PCR is correlated with the size of the amplicon, which can result in preferential amplification of smaller products.

Reverse Transcriptase PCR

In reverse transcriptase PCR (RT-PCR), cDNA copies are made of RNA, followed by normal PCR amplification of the desired target.

Real-Time PCR

In real-time PCR, the production of amplicons is measured in "real time" during the amplification process (6). Numerous methods have been described, ranging from the use of nonspecific staining of double-stranded DNA using intercalating dyes to the use of fluorescence-labeled DNA probes (7). One of the most commonly used probe-based chemistries is the use of hydrolysis or TaqMan probes (Fig. 1A), in which the 5′-to-3′ exonuclease activity of *Taq* polymerase cleaves the hybridized probe during the elongation phase of the amplification reaction. In this process, the fluorescent molecule at the 5′ end of the probe is separated from the quencher molecule at the 3′ end of the probe, resulting in a fluorescent signal that can be measured after each amplification cycle. Amplification can also be detected by using fluorescence resonance energy transfer (FRET) probes (Fig. 1B), which comprise two adjacent hybridizing probes that are labeled at the 3′ and 5′ ends of the probe with a donor and an acceptor fluorescent molecule, respectively. An additional melt curve analysis (see below) can be used for the detection of point mutations in the probe sites, which can provide additional differentiation of the sequence detected. Another example of probe-based real-time PCR chemistry is scorpion probes (or primers), which are composed of a primer region with a covalently linked probe and a self-complementary stem sequence with a 5′ fluorophore and a 3′ quencher (Fig. 1C). In the amplification process, the loop sequence hybridizes with the complementary internal target sequence, separating the reporter from the quencher and allowing the reporter to fluoresce.

The amplification cycle at which the level of fluorescent signal exceeds the background fluorescence (threshold cycle [C_T] value) is directly correlated with the initial amount of target DNA in the sample, making quantification possible. The absence of a postamplification process reduces the risk of contamination, labor time, and reagent costs. Separate measurement of probes with different fluorophores emitting fluorescence at different wavelengths enables the implementation of multiplex PCRs of similar-sized DNA fragments with the same efficiency.

High-Resolution Melt Curve Analysis

In high-resolution melt curve (HRM) analysis, the decrease of fluorescence of an intercalating dye is measured in the process of the separation of double-stranded DNA by a gradual increase in temperature. Differences in the melting temperature (T_m) of an amplicon reflect differences in the nucleotide sequence.

PCR-Restriction Fragment Length Polymorphism

In PCR-restriction fragment length polymorphism (PCR-RFLP) analysis, PCR products are digested with restriction enzymes to produce different numbers and sizes of fragments depending on

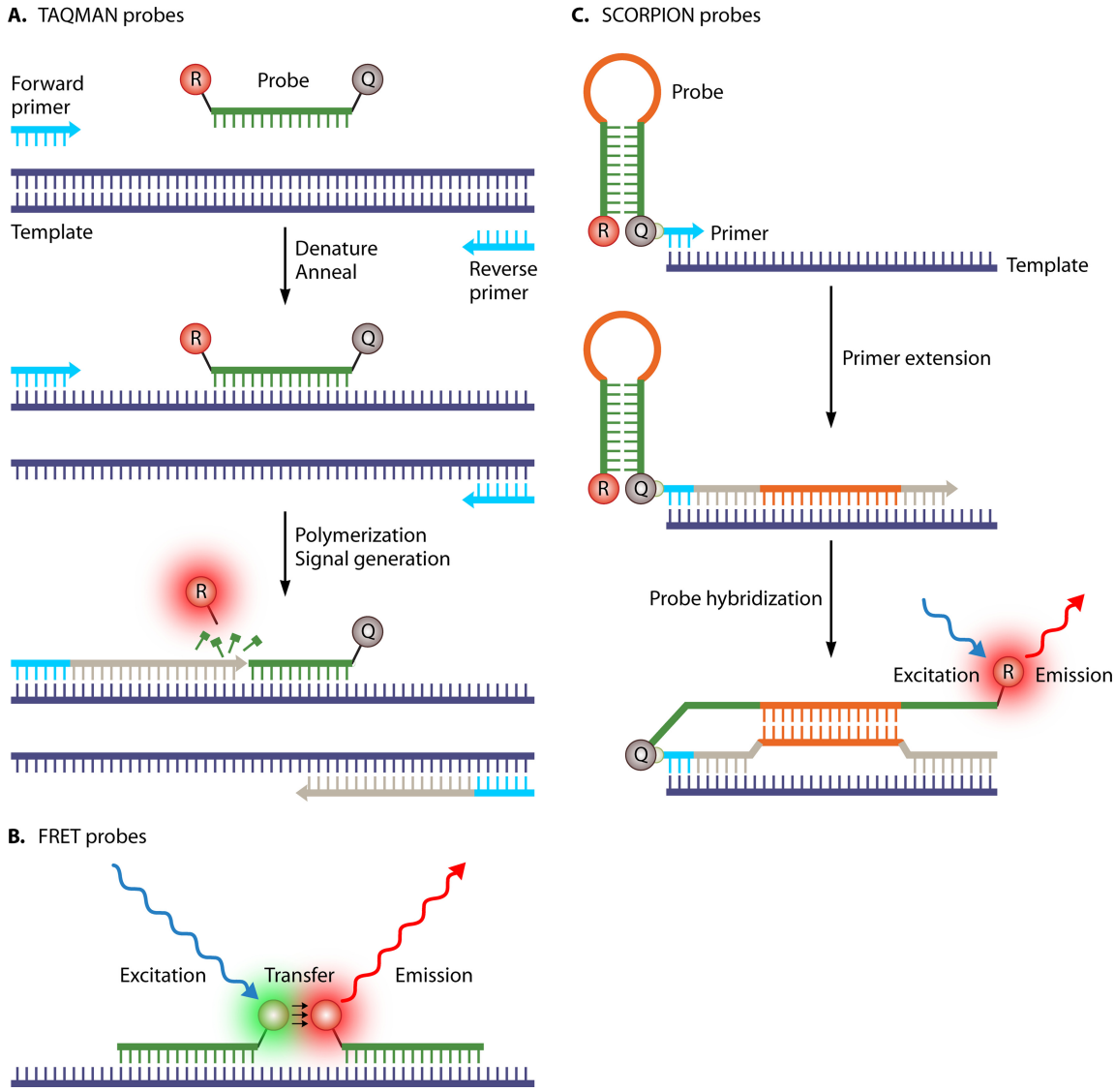

FIG 1 Three examples of probe-based real-time PCR chemistry. (A) Hydrolysis or TaqMan probes; (B) fluorescence resonance energy transfer (FRET) probes; (C) scorpion probes.

differences in the number and location of restriction sites in the amplicon. Target genes and restriction enzymes are chosen in a way that, for example, different species within a genus produce the same-sized amplicon but reveal different banding patterns by gel electrophoresis after digestion.

Random Amplified Polymorphic DNA

Random amplification of polymorphic DNA (RAPD) is performed by using single primers with arbitrarily chosen short nucleotide sequences to amplify products from genomic DNA. After optimization, genus-, species-, or strain-specific banding patterns representing different DNA regions throughout the whole genome can be obtained. The use of these nonspecific primers requires DNA from a pure isolate without contamination by DNA from other organisms, which makes it impossible to use this method on genomic DNA isolated from clinical samples. Specific DNA products of interest can be isolated, sequenced, and used for the development of specific assays targeting these products (8).

Amplification Fragment Length Polymorphism

The amplification fragment length polymorphism (AFLP) technique is based on selective amplification of restriction fragments derived from digested genomic DNA. DNA is digested with restriction enzymes, and oligonucleotide adapters are ligated to the restriction fragments. Thereafter, PCR is used for selective amplification of the restriction fragments, and the amplified fragments are separated by gel or capillary electrophoresis. Similar to the case for RAPD, genus-, species-, or strain-specific banding patterns can be obtained provided that pure isolates are available.

Single-Strand Conformation Polymorphism

Single-strand conformation polymorphism (SSCP) is a mutation-scanning method based on the differential migration in gel electrophoresis of single-stranded DNA molecules of the same size but with different conformations due to differences in the nucleotide sequences.

Multiplex Ligation-Dependent Probe Amplification

Multiplex ligation-dependent probe amplification (MLPA) uses two target-specific oligonucleotides (probes) for each target of interest with a universal primer sequence on the 5′ end of one probe and a sequence of a known length assigned to the target of interest (stuffer sequence) followed by a universal primer sequence on the 3′ end of the second probe. The two hybridized probes are joined together through ligation using a DNA ligase enzyme. The resulting product can be amplified by using primers targeting the universal primer sequences on both ends of the product. The PCR products can be separated on the basis of the different lengths of the unique stuffer sequences using gel or capillary electrophoresis, enabling multiplex detection of a large number of targets (9).

PCR-Reverse Line Blot Assay

In PCR-reverse line blot (RLB) analysis, biotin-labeled primers are used to produce a biotin-labeled PCR product and probes that are covalently bound to a membrane. The biotin-labeled PCR product is hybridized to the probes on the membrane, and the hybridization product is visualized on an X-ray film by a biotin-streptavidin-peroxidase-mediated chemiluminescence reaction.

Loop-Mediated Isothermal Amplification

Loop-mediated isothermal amplification (LAMP) typically uses four target-specific oligonucleotides to amplify DNA under isothermal conditions (10). The amplified DNA product can be detected by the naked eye as a white precipitate in the reaction tube or under UV light after the addition of a fluorescent intercalating dye.

Multilocus Sequence Typing

For multilocus sequence typing (MLST), depending on the degree of discrimination required, a number of housekeeping genes of an isolate are amplified and sequenced on both strands. Each sequence variant within each gene is assigned to a distinct allele, and the combination of alleles within an isolate defines its allelic profile or sequence type (SQT).

Basic Local Alignment Search Tool

The Basic Local Alignment Search Tool (BLAST) is an online algorithm that can be used to compare DNA sequences of interest against several DNA sequence databases.

DNA ISOLATION

Without an appropriate nucleic acid isolation method, DNA amplification techniques will not be reliable. Basically, there are two essential points that must be kept in mind. First, will the isolation method used be able to release the nucleic acids from the parasitic stage (e.g., cysts, spores, or eggs), which is to be expected in the clinical sample? Second, will the nucleic acids that are isolated be free of substances that may interfere with or inhibit the amplification reaction? It is not hard to imagine that the latter is especially important in the isolation of parasite DNA from a complex matrix such as feces (11). Heating of the stool specimen and the addition of absorbent substances such as polyvinyl polypyrrolidone during the DNA isolation procedure or the addition of inhibition factor-binding substances, such as bovine serum albumin (BSA), or inhibitor-resistant DNA polymerases in the PCR mixture can be used to prevent inhibition of the amplification reaction (12–15). However, it remains important to include an internal inhibition control in each reaction mixture. For example, phocin herpesvirus (PhHV) is frequently used and is added to each sample for the isolation procedure, after which a PhHV-specific PCR within the multiplex of the respective targets is performed (16). Because the same amount of virus is added to each sample, it is expected that the fluorescent signal of the PhHV-specific probe will cross the threshold at the same amplification cycle for each sample (C_T value). If a higher or no C_T value is found in a sample, it suggests inhibition of the amplification reaction. In such cases, DNA isolation and PCR should be repeated using a diluted sample. With regard to the first aspect, it appears that the efficient release of nucleic acids depends on a balanced combination of actions in the DNA isolation procedure. For example, some papers mention specifically that additional mechanical disruption is needed for the efficient isolation of DNA from *Trichuris* eggs or *Entamoeba* cysts, while others use a standard isolation protocol without mention of additional rigorous steps to break down the egg shell or cyst wall (15, 17–19). Another example is the negative effect of preserving feces in formalin or sodium acetate-acetic acid-formalin (SAF) on the specific amplification of *Entamoeba*, *Giardia*, and *Cryptosporidium* DNAs; this effect increases with the duration of fixation (20–23). Species-specific DNA extraction using magnetic beads which capture oligonucleotides specific for *Cryptosporidium* and *Giardia* improved (lowered) real-time PCR C_T values by averages of 10.7 and 9.7 cycles, respectively (24). Nowadays, more and more commercially available DNA isolation kits and protocols for automated DNA isolation systems are becoming available for DNA isolation from feces (25–27). The efficiency of DNA isolation for each target in the expected variety of clinical samples should be verified when such systems are introduced.

ASSAY DEVELOPMENT AND VALIDATION

Extensive knowledge of genetic variation across species and genera is paramount in efforts to tailor DNA-based assays to relevant organisms. Alignments of target sequences from related and unrelated organisms are used to aid the design of organism-specific detection assays, and a choice is made of a target sequence that is specific for the organism of interest and does not show sequence variation within the organism. For example, it is important to know that *Cryptosporidium* assays based on the DnaJ-like sequence, which are quite commonly used in diagnostic settings, will most likely detect *C. hominis* and *C. parvum* only, and as a consequence, other rarer *Cryptosporidium* species will remain undetected. This can be overcome by using an assay based on the small-subunit (SSU) rRNA gene, but it should be taken into account that the SSU rRNA gene is highly conserved among some apicomplexan genera. Thus, other related species, such as *Cyclospora* and *Cystoisospora*, could be detected as well. Obviously, in the design of genotype-specific detection assays, target sequences that show genotype-specific variation within the organism are used to design genotype-specific primers and probes (28).

Sequence data that are used for the design and evaluation of diagnostic primers and probes using BLAST searches are limited mainly to data available in GenBank (28). Moreover, as pointed out by Stensvold et al. (28) and Burnet et al. (29), the nomenclature in GenBank does not always follow the changing taxonomy, and there are several falsely annotated sequences, so care should be taken when designing primers and interpreting PCR results,

whether *in silico* (i.e., using software to analyze and predict PCR outcomes) or in clinical situations.

Such evaluations should not be performed only when a diagnostic assay is introduced but have to be maintained through continuous monitoring of the literature. While *in silico* evaluation of primer/probe specificity and sensitivity is a key component in the development, integration, and validation of diagnostic assays, extensive laboratory testing using panels of control DNA samples of individual worms, cultured or purified organisms, and isolated fecal genomic DNAs may further challenge the sensitivity and specificity of any given assay. For instance, the SSU rRNA gene is highly conserved among some apicomplexan genera, but the lack of specificity is somewhat balanced by the fact that many of these genera are parasites of nonhuman hosts (28).

PROTISTS

More than 15 protistan genera are known to parasitize the human intestine. For some genera, only one species has been found in humans, for instance, *Balantidium*, while humans are natural hosts of several species of *Entamoeba*, for instance. Human intestinal protists each belong to one of six biologically very different groups: amoebozoans, metamonads, ciliates, apicomplexans, microsporidia, and stramenopiles. Microsporidia and the Apicomplexa are all obligate intracellular parasites and as such are directly responsible for tissue damage that often leads to symptoms in the infected host. Asymptomatic carriage is not uncommon for most parasitic infections; however, treatment of even asymptomatic carriers of, for instance, *Entamoeba histolytica* and *Giardia* is important to reduce transmission and episodes of recrudescence. In this review, only species that have been positively linked to disease are included. A positive link means that the parasite may cause dysentery (e.g., *Entamoeba histolytica* and *Balantidium coli*) and/or has been identified as a recurring cause of outbreaks of diarrhea (e.g., *Cryptosporidium*, *Cyclospora*, and *Giardia*) or multiple sporadic cases of diarrhea, for instance, in immunocompromised individuals (e.g., microsporidia and *Cystoisospora belli*).

In addition to these organisms, *Blastocystis* and *Dientamoeba fragilis* have been included, although the pathogenicity of these organisms is the subject of ongoing debate. However, without the routine use of permanently stained fecal smears, these parasites are notoriously difficult to detect, and the use of nucleic acid-based techniques has largely facilitated both detection and differentiation. Thanks to these techniques, we are starting to build a picture of their epidemiology and clinical significance, which is why we have chosen to include them.

AMOEBOZOA

Entamoeba

Entamoeba histolytica is the causative agent of amoebic dysentery and is able to penetrate the gut wall and reach the liver, where it can cause severe damage by lysing liver tissue (30). Worldwide, an estimated 50 million people are infected with *E. histolytica*, and 40,000 people die annually from the consequences of this infection (31).

In 1997, the existence of *E. histolytica* and *Entamoeba dispar* as two distinct but morphologically identical species was officially acknowledged, with only the former causing disease and the latter being regarded as a harmless intestinal commensal (30, 32). The global epidemiology of *E. histolytica* and *E. dispar* has been reviewed quite extensively, showing large variations in the ratios between *E. histolytica* and *E. dispar* in different regions (33, 34). Studies of travelers and immigrants performed in laboratories situated in countries where these organisms are not endemic have shown a 1:10 *E. histolytica*/*E. dispar* ratio (35–38). which is in agreement with the assumed worldwide ratio (39).

In nondiarrheic stool samples, *Entamoeba* cysts may be identified by, e.g., microscopy of fecal concentrates. Several species of *Entamoeba* (e.g., *E. histolytica*, *E. dispar*, *E. moshkovskii*, *E. coli*, *E. hartmanni*, and *E. polecki*) are capable of establishing infection in the human intestine, and these species can be separated in part based on morphological analysis of cysts. Mature cysts from *Entamoeba* species infecting humans are mainly uni-, quadri-, or octanucleated. *E. histolytica* produces quadrinucleated cysts, which are generally morphologically indistinguishable from cysts produced by some nonpathogenic species of *Entamoeba*. Demonstration of erythrocytes within trophozoites of *E. histolytica* (Fig. 2A) is diagnostic but requires the availability of highly specialized personnel and access to freshly passed dysenteric stool samples or the use of fecal fixatives for specimen submission (40). Stool antigen assays appear to be specific and sensitive for the detection of *E. histolytica* infections in areas of endemicity (41) but lack sensitivity in settings where the disease is not endemic (42, 43).

Apart from *E. histolytica* and *E. dispar*, two additional species producing quadrinucleated cysts, *E. moshkovskii* and *E. bangladeshi*, have been identified in humans. *E. moshkovskii* has been detected frequently by using nested PCRs in mixed infections with *E. histolytica* and/or *E. dispar* (Table 1). Although *E. moshkovskii* is regarded mostly as nonpathogenic, it has been associated with gastrointestinal complaints in some studies (44–47). Little is yet known about the genetic presence of virulence factors or the expression thereof in comparison with *E. histolytica*. The biology, diagnosis, epidemiology, and clinical aspects of infections with *E. moshkovskii* were recently reviewed (48).

Recently, *E. bangladeshi* was identified in stool samples from children in Bangladesh (49). A genus-specific primer pair (50) was used to analyze fecal DNAs from Bangladeshi children with and without diarrhea who were microscopy positive for quadrinucleated cysts but PCR negative for *E. histolytica*, *E. dispar*, and *E. moshkovskii*. Sequencing of PCR products produced evidence of a novel species, named *E. bangladeshi*. The morphology of cysts and trophozoite stages of *E. bangladeshi* appears similar to that of *E. histolytica* (49, 51). Phylogenetic analysis of the relationship between *E. bangladeshi* and other *Entamoeba* parasites reveals that, although distinct, *E. bangladeshi* clearly groups with the clade of *Entamoeba* parasites infecting humans, which includes *E. histolytica*, *E. dispar*, and *E. moshkovskii*; this "complex" moreover contains two additional species that have not been found in humans so far, namely, *E. ecuadoriensis* (sewage) and *E. nuttalli* (nonhuman primates [NHPs]) (52, 53). Of note, *E. nuttalli* differs from *E. histolytica* by 2 to 3 bp precisely in the DNA sequence of the *E. histolytica* detection probe of a widely used real-time PCR based on SSU rRNA genes of *E. histolytica* and *E. dispar* (28). As a result, DNA amplification will occur but will probably not be detected by this assay. Whereas *E. nuttalli* appears to be virulent in nonhuman primates, at present, it remains unclear whether *E. nuttalli* can infect and cause disease in humans (53–57).

The genetic universe of *Entamoeba* is currently rapidly expanding, which is due mainly to the recent application of sequencing of PCR products amplified from DNA extracted directly from feces.

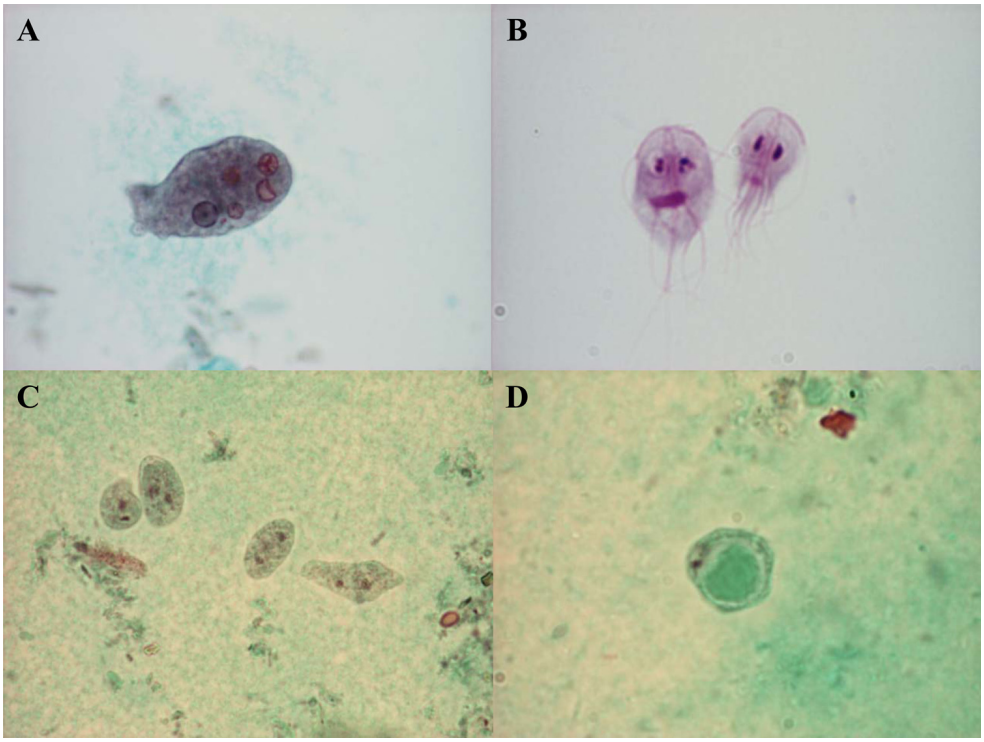

FIG 2 Trichrome staining of an *Entamoeba histolytica* trophozoite with ingested red blood cells (A), *Dientamoeba fragilis* trophozoites (C), and the *Blastocystis* vacuolar stage (D). (B) Giemsa staining of *Giardia lamblia* trophozoites. (Parasite images courtesy of Marianne Lebbad; reprinted with permission.)

This has resulted in the discovery of a large number of novel *Entamoeba* lineages and subtypes (STs) from human and nonhuman hosts. To date, at least 29 distinct lineages have been identified (50, 58). The search for and ongoing discovery of such new lineages warrant substantial additional sampling in order to identify the clinical significance of each lineage and to enable further intrageneric analysis of *Entamoeba*.

The absence of morphologically apparent differences has made it necessary to develop new diagnostic techniques both for diagnosis and for an understanding of the epidemiology of *E. histolytica* infections. Laboratory techniques for the diagnosis of *E. histolytica* infections have been reviewed in detail (59, 60). A range of DNA targets, single- and multicopy genes, have been used in single-round and nested conventional PCRs and real-time PCRs. More recent assays (35, 61–64) using (multiplex) real-time PCR or (pyro)sequencing have been based on the SSU rRNA gene, which appears to be the best diagnostic target in terms of sensitivity and specificity compared to real-time PCR assays targeting non-SSU rRNA genes (65). A method for the detection and differentiation of five species of *Entamoeba*, including *E. histolytica*, based on Luminex technology was recently developed (66). One of the species targeted was *Entamoeba coli*, which exhibits remarkable intraspecies variation, and currently, two subtypes are known (ST1 and ST2) (50). Meanwhile, the probes used for detection of *E. coli* by Santos et al. (66) were identical only to ST1 and might not detect *E. coli* ST2, which is currently represented in GenBank under accession numbers AF149914 and AB444953. While the approach is appealing, the challenges and limitations of basing diagnostics exclusively on detection probes are evident.

Genetic variation within *E. histolytica* in protein-coding genes, noncoding genes, and short tandem-repeat (STR) loci was reviewed by Ali et al. (33). Although one study in Bangladesh has shown a link between genotypes and symptoms, it appears that most strains of *E. histolytica* have the ability to invade host tissue and cause disease. While the overall genetic diversity of *E. histolytica* based on single-nucleotide polymorphisms (SNPs) appears to be low, there is substantial genetic variability in highly repetitive DNA regions and across SNPs in some coding genes. A multilocus sequence typing (MLST) system was recently developed based on 16 polymorphic loci identified by next-generation sequencing of *E. histolytica* genomes and verified by Sanger sequencing (67). As this system is based on SNPs in protein-coding genes potentially involved in pathogenesis rather than on surrogate markers such as tRNA STR patterns, this method may hold promise for further clinical and epidemiological investigations. However, it remains unclear whether the extensive genetic diversity seen among the isolates analyzed reflects a high rate of evolution by recombination or reassortment events that may drive any observed differences between *E. histolytica* genotypes in samples isolated from the same geographical area. Hence, although a couple of SNPs in the cyclin-2 locus appeared to be associated with disease, it is not yet clear whether differences in SNPs are associated with differences in clinical outcomes or geographical differences. In summary, genotyping currently has very limited clinical significance and is not used in a diagnostic setting but for epidemiology research only.

PCRs for the detection and differentiation of *E. histolytica* and *E. dispar* were probably some of the first parasite PCRs to be widely introduced into routine diagnostic laboratories and are now typically used in situations where quadrinucleated cysts have been found in stool samples for species identification, sometimes in addition to serology testing. PCR technology also facilitates efforts

TABLE 1 PCR-based studies that have included PCR detection of *E. moshkovskii* rDNA[h]

Country	Method	No. of patients	Sample selection criterion	No. of samples tested	% positive samples				Reference(s)
					E. histolytica[a]	*E. dispar*[a]	*E. moshkovskii*[a]	*E. moshkovskii* only	
Bangladesh	Nested PCR	109		109	16	36	21	6	427
Ghana	PCR-RLB	246		20	0	45	0		428
India	Nested PCR-RFLP[c]	746	Microscopy positive	68	19	97	25	1	429
Thailand	Multiplex PCR		Microscopy positive	30	13	20	0	0	430
Iran	Nested PCR[d]	1,037	Microscopy positive	88	0	100	1	1	431
India	Nested PCR-RFLP[d]	1,720	Microscopy positive/ culture positive	202	30	79	16	1	432
India	Nested multiplex PCR	1,720	Microscopy positive/ culture positive	202	35	84	18	0	433
Turkey	Nested PCR-RFLP[d]	100	Diarrhea cases	100	23[c]	NA	2	NA	434
Australia	Nested PCR[d]	1,246	MSM/microscopy positive	54	5	65	31	15	44
Australia	Nested PCR[d]	5,921	Microscopy positive	110	5	57	50	24	435, 436
Tunisia	Nested PCR[d]		Microscopy positive	27	0	89	7	0	437
India	Nested multiplex PCR[e]		Microscopy positive	202	35	85	18	NA	438
Tanzania	Nested PCR[d]	136	HIV positive	136	4[c]	5	13	12	439
		622	Controls		0	0	0	0	
Thailand	Multiplex real-time (FRET) PCR		Microscopy positive	33	6	85	1	0	61
India	Nested multiplex PCR[e]	246	Microscopy positive	49	12	29	8	NA	440
NA[i]	PCR-pyrosequencing	NA	Microscopy positive/ *E. histolytica-E. dispar* PCR positive	102	17	86	0	0	64
Iran	PCR[f]	3,825	Microscopy positive	58	3	93	5	2	441
Brazil	PCR-sequencing		Microscopy positive/ culture positive	29	14	66	3[b]	3[b]	63
Ecuador	PCR-RLB[g]	674	Microscopy positive	101	0	8	0	0	442
Pakistan	Nested multiplex PCR[e]	129	Diarrhea cases	129	9	19	19	NA	45
		151	Controls	151	1	27	4	NA	
Malaysia	PCR[f]	500		500	6	17	3	1	46, 443–445
Malaysia	Nested multiplex PCR[f]	426		426	9	4	1	1	446

[a] Including mixed infections.
[b] DNA isolated from culture.
[c] Antigen detection only.
[d] PCR primers are described in reference 427.
[e] PCR primers are described in reference 433.
[f] PCR primers are described in reference 430.
[g] PCR primers are described in reference 428.
[h] Abbreviations: RLB, reverse line blot; RFLP, restriction fragment length polymorphism; FRET, fluorescence resonance energy transfer; MSM, men who have sex with men; NA, not available.
[i] Samples from the indicated study were from patients that were tested in Sweden, Denmark, and The Netherlands.

toward identification of *E. histolytica* in samples other than stool, e.g., aspirates from liver abscesses, cerebrospinal fluid (CSF), and urine (51, 68–70). The wider availability of real-time PCR platforms has paved the way for the routine use of PCRs for this and other parasite targets as first-line diagnostic methods. Indeed, *E. histolytica*-specific gene targets have been integrated into several recent high-throughput multiplex assays aiming to detect a steadily increasing number of enteropathogens (Table 2).

METAMONADS

Giardia

The genus *Giardia* comprises multiple species whose taxonomy is in a state of flux. *Giardia lamblia* (synonyms, *G. duodenalis* and *G. intestinalis*) (Fig. 2B) is one of the most common intestinal parasites of humans (71); in some cohorts, for instance, Cuban children, the prevalence may exceed 50% (72). Transmission is by the fecal-oral route, and the parasite has a simple life cycle comprising excystation in the duodenum; colonization by rapidly multiplying, noninvasive trophozoites on the mucosal surface of the small intestine; and, eventually, the production of environmentally resistant cysts that are shed with host feces. Food- and waterborne outbreaks have frequently been reported (73). Symptoms may include late-onset and persistent diarrhea (rather than acute diarrhea), abdominal cramps, bloating, steatorrhea, malabsorption, weight loss, and stunting (74–76). Stools from symptomatic carriers may be mushy, greenish, and foul-smelling.

Persistent symptoms due to, or following, *Giardia* infection take a significant toll on human health. Reappearance of symptoms, including abdominal symptoms and fatigue, can result from reinfection, recrudescence, perturbation of the gut flora, or

TABLE 2 Examples of multiplex pathogen detection approaches including parasitic targets[m]

Assay format	Combined assay targets	Sample selection criterion	No. of samples tested	Commercial availability	Description	Reference(s)
Multiplex real–time PCR[a]	*E. histolytica*, *C. parvum sensu lato*,[l] *G. lamblia*, and PhHV	Microscopy–positive stool samples	112	In–house	First multiplex real–time PCR described for detection of diarrhea–causing protozoa; has been adapted in many diagnostic laboratories since then	79
Multiplex PCR array	*E. histolytica*, *E. dispar*, *Cryptosporidium*, and *G. lamblia*	Control DNA only		In-house	Simultaneous detection, species differentiation, and genotyping after hybridization of a multiplex PCR in a microarray format	447
Multiplex real-time PCR[b]	*G. lamblia*, *Cryptosporidium*, and *E. histolytica*	Antigen- and PCR-positive stool samples	129	In-house	Primers designed from the COWP gene should be able to detect *C. parvum*, *C. hominis*, and *C. meleagridis* but not other species of *Cryptosporidium*	448
Multiplex real-time PCR[c]	*E. histolytica*, *C. parvum sensu lato*, *G. lamblia*, and PhHV	Gastrointestinal complaints	956	In-house	Higher detection rates found for *Giardia* and *Cryptosporidium* with PCR, and no additional parasites found with microscopy in a general practitioner patient population	104
Multiplex real-time PCR	*N. americanus*, *A. duodenale*, *O. bifurcum*, and PhHV	No selection, population based	339	In-house	Good correlation found between DNA load and egg/larval counts	328
Multiplex real-time PCR[d]	*E. histolytica*, *C. parvum sensu lato*, *G. lamblia*, and PhHV; *S. stercoralis* and PhHV	Stool samples from travelers	2,591	In-house	PCR for the targets used outperformed expert microscopy; strikingly, even in travelers, not many additional parasites were found by microscopy	107
Multiplex real-time PCR[d,e]	*E. histolytica*, *C. parvum sensu lato*, *G. lamblia*, and PhHV; *D. fragilis* and PhHV	Stool samples from patients with gastrointestinal complaints	397	In-house	Increased detection rate of *D. fragilis* with PCR (31%) compared to that with microscopy (17%)	27
Multiplex real-time PCR[b]	*G. lamblia*, *Salmonella enterica*, *Campylobacter jejuni*, and PhHV	Stool samples from patients with gastrointestinal complaints	13,974	In-house	Implementation of *Giardia* PCR in a routine diagnostic laboratory	106
Multiplex real-time PCR[f,g]	*A. duodenale*, *N. americanus*, *A. lumbricoides*, *S. stercoralis*, and PhHV	No selection, population based	1,312	In-house	Large albendazole placebo-controlled trial on the effect of STH infections on allergy, atherosclerosis, and malaria; effect of treatment was monitored by quantitative real-time PCR	319, 321, 333, 449
Multiplex nested PCR-RFLP	Microsporidia, *Cyclospora*, and *Cryptosporidium*	Controls		In-house	Conventional and nested PCR are not very practical in a routine setting	450
Multiplex tandem real-time PCR	*E. histolytica*, *Cryptosporidium*, *G. lamblia*, and *D. fragilis*	Stool samples from patients with gastrointestinal complaints	472	AusDiagnostics, Beaconsfield, Australia	Automated system of multiplex preamplification followed by target-specific real-time PCR	451
Multiplex PCR using Luminex beads[b,f,g,h,i]	*E. histolytica*, *Cryptosporidium*, *G. lamblia*, *A. duodenale*, *N. americanus*, *A. lumbricoides*, and *S. stercoralis*	Microscopy- and/or PCR-positive stool samples	319	In-house	Luminex detection of multiplex PCR products using primers and probes that were described previously as real-time TaqMan-based assays	322

Multiplex PCR using Luminex beads	*C. cayetanensis*, *C. belli*, *E. bieneusi*, and *E. intestinalis*	Microscopy- and/or PCR-positive stool samples	234	In-house	Luminex detection of multiplex PCR products using primers and probes that were described previously as real-time TaqMan-based assays	193
Multiplex real-time PCR[f,g,h]	*A. duodenale*, *N. americanus*, *A. lumbricoides*, *S. stercoralis*, and PhHV	Stool samples from patients with gastrointestinal complaints	78	In-house	Application of multiplex real-time PCR in Malaysia for STH infection, finding higher detection rates with PCR, especially for *A. lumbricoides* and *S. stercoralis*	320
Multiplex real-time PCR[c,e,j]	*E. histolytica*, *E. dispar*, *C. parvum sensu lato*, and *G. lamblia*; *E. bieneusi* and *Encephalitozoon* spp.; *D. fragilis*; *Blastocystis*	HIV positive	96	In-house	Although a high prevalence of intestinal parasites was detected, there was no association between any of the parasites and the presence of diarrhea	160
Multiplex tandem real-time PCR	*Campylobacter*, *Clostridium difficile*, *Salmonella*, *Cryptosporidium*, *G. lamblia*, *Shigella*, adenovirus 40/41, and norovirus	*Cryptosporidium*-positive samples and negative-control samples	267	AusDiagnostics	Extension of the no. of targets[n]	452
Multiplex real-time PCR[c,f,g,h]	*E. histolytica*, *Cryptosporidium*, and *G. lamblia*; *A. duodenale*, *N. americanus*, *A. lumbricoides*, and *S. stercoralis*	Stool samples from patients with gastrointestinal complaints	229	In-house	Application of multiplex real-time PCR in Malaysia for STHs and diarrhea-causing protozoa	161
Multiplex PCR	*E. histolytica*, astrovirus, calicivirus, and EIEC	Stool samples from patients with gastrointestinal complaints	103	In-house	Conventional PCR is not very practical in a routine setting with large numbers of samples	453
Multiplex real-time PCR[c,e]	*E. histolytica*, *C. parvum sensu lato*, and *G. lamblia*; *E. dispar*; *D. fragilis*	Stool samples from patients with gastrointestinal complaints	889	In-house	PCR showed higher detection rates of the parasites targeted; no additional pathogens were found by FECT-microscopy	162
TaqMan open array	*Cryptosporidium*, *G. lamblia*, *Campylobacter* spp., *Clostridium difficile*, *Salmonella*, *Vibrio parahaemolyticus*, diarrheagenic *Escherichia coli* (EHEC), *Shigella*, *Yersinia enterocolitica*, *Listeria monocytogenes*, adenovirus, astrovirus, norovirus GI, norovirus GII, rotavirus, and sapovirus	Stool samples from patients with gastrointestinal complaints	86	In-house	A DNA sample with PCR mix is distributed by hydrophilic forces into 64 through-holes, in which primers and a probe are spotted by the manufacturer; in this way, up to 64 PCRs per sample can be performed for 48 samples simultaneously	87, 454
Multiplex real-time PCR[d]	*E. histolytica*, *C. parvum sensu lato*, *G. lamblia*, and PhHV	Stool samples from patients with gastrointestinal complaints	396	In-house	Microscopy showed low sensitivity and specificity compared to real-time PCR, especially for detection of *E. histolytica*	87

(Continued on following page)

TABLE 2 (Continued)

Assay format	Combined assay targets	Sample selection criterion	No. of samples tested	Commercial availability	Description	Reference(s)
TaqMan array card	*Cryptosporidium*, *G. lamblia*, *Ascaris lumbricoides*, *Trichuris trichiura Campylobacter jejuni-C. coli*, *Clostridium difficile*, *Salmonella*, *Vibrio cholerae*, diarrheagenic *Escherichia coli* (EAEC, ETEC, EPEC, and STEC), *Shigella*/EIEC, adenovirus, astrovirus, norovirus GII, rotavirus, sapovirus, and PhHV	Microscopy-, culture-, and/or immunoassay-positive stool samples	109	In-house	A DNA sample with PCR mix is distributed by centrifugation into 42 wells of a microfluidic card in which primers and probe are spotted by the manufacturer	309
TaqMan panel[f,i]	*E. histolytica*, *Cryptosporidium*, *G. lamblia*, *A. duodenale*, *N. americanus*, *A. lumbricoides*, *S. stercoralis*, and *T. trichiura*	No selection, population based	525	In-house	Parallel testing of 8 singleplex PCR mixtures that were prealiquoted and stored frozen until use; good correlation between egg counts and DNA load; all *Ascaris* PCR-positive cases tested negative 3 wk after treatment	18
Multiplex real-time PCR	*E. histolytica*, *Cryptosporidium*, and *G. lamblia*; *Campylobacter coli-C. jejuni*, *E. coli* (VTEC), *Salmonella* spp., *Shigella* spp., *E. coli* (EIEC), *Y. enterocolitica*, and *C. difficile*; norovirus GI, norovirus GII, and IC; adenovirus, astrovirus, and rotavirus	Stool samples from patients with gastrointestinal complaints	1,758	CE labeled (Fast-Track Diagnostics, Luxembourg)	Enhanced rate of detection of diarrhea-causing pathogens using a broad approach due to more sensitive detection of pathogens and detection of pathogens that were not requested	413
Multiplex PCR using Luminex beads	*E. histolytica*, *Cryptosporidium*, *G. lamblia*, *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *E. coli* O157, ETEC, STEC, *C. difficile*, *Y. enterocolitica*, *V. cholerae*, norovirus GI, norovirus GII, adenovirus 40/41, and rotavirus A	Stool samples from patients with gastrointestinal complaints	901	FDA cleared, CE labeled (Luminex)	Multicenter study comparing standard routine procedures including culture, antigen tests, and real-time PCR with Luminex detection of multiplex PCR products of 15 viral, bacterial, and parasitic pathogens (Luminex xTAG GPP)	423
Multiplex PCR using Luminex beads	*E. histolytica*, *Cryptosporidium*, *G. lamblia*, *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *E. coli* O157, ETEC, STEC, *C. difficile*, *Y. enterocolitica*, *V. cholerae*, norovirus GI, norovirus GII, adenovirus 40/41, and rotavirus A	Stool samples from diarrheic patients	440	FDA cleared, CE labeled (Luminex)	Luminex xTAG GPP was compared with standard routine procedures which did not include real-time PCR; no further analysis of discrepant results	422

Multiplex PCR using Luminex beads	*E. histolytica*, *Cryptosporidium*, *G. lamblia*, *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *E. coli* O157, ETEC, STEC, *C. difficile*, *Y. enterocolitica*, *V. cholerae*, norovirus GI, norovirus GII, adenovirus 40/41, rotavirus A	Stool samples from patients with gastrointestinal complaints	393	FDA cleared, CE labeled (Luminex)	Luminex xTAG GPP was compared to standard routine procedures including culture, antigen tests, and real-time PCR; 5 of 6 *E. histolytica*-positive results by xTAG GPP could not be confirmed	455
Multiplex real-time PCR[f,g,j,k]	*A. duodenale*, *N. americanus*, *A. lumbricoides*, *S. stercoralis*, and PhHV; *Schistosoma* and PhHV	Stool samples from HIV-positive patients	153	In-house	Improved detection of helminth infections using real-time PCR; discrepancies in risk factor analysis between qualitative data from PCR and microscopy; this was resolved by using quantitative data from PCR	456

[a] PCR primers and probe for *Cryptosporidium* are described in reference 158.
[b] PCR primers and probe for *Giardia* are described in reference 79.
[c] PCR primers and probes for *E. histolytica*, *Cryptosporidium*, and *Giardia* are described in reference 79.
[d] PCR primers and probes for *E. histolytica* and *Giardia* are described in reference 79.
[e] PCR primers and probe for *D. fragilis* are described in reference 128.
[f] PCR primers and probes for *N. americanus* and *A. duodenale* are described in reference 328.
[g] PCR primers and probe for *S. stercoralis* are described in reference 354.
[h] PCR primers and probe for *A. lumbricoides* are described in reference 319.
[i] PCR primers and probe for *E. histolytica* are described in reference 448.
[j] PCR primers and probes for *E. bieneusi* and *Encephalitozoon* spp. are described in reference 457.
[k] PCR primers and probe for *Schistosoma* are described in reference 458.
[l] Nomenclature of the sequence from GenBank used for the design of primers and probes was for *C. parvum*, which was later separated into *C. hominis* and *C. parvum*.
[m] Abbreviations: RFLP, restriction fragment length polymorphism; SSU, small subunit; ITS, internal transcribed spacer; STH, soil-transmitted helminth; GPP, gastrointestinal pathogen panel; COWP, *Cryptosporidium* oocyst wall protein; FECT, formol ethyl-acetate concentration technique; EIEC, enteroinvasive *Escherichia coli*; EHEC, enterohemorrhagic *E. coli*; EAEC, enteroaggregative *E. coli*; ETEC, enterotoxigenic *E. coli*; EPEC enteropathogenic *E. coli*; STEC, Shiga-toxigenic *E. coli*; VTEC, verotoxin-producing *Escherichia coli*; IC, internal control; CE, European Community; PhHV, phocine herpesvirus.
[n] As described in reference 451.

postinfection syndromes (77). In developed countries, such sequelae may have a vast impact on quality of life; in developing countries, particularly in children, they add yet another burden to already disadvantaged populations (77).

Several conventional and real-time PCRs for the primary diagnosis of giardiasis that target the SSU ribosomal DNA (rDNA), β-giardin, triosephosphate isomerase (TPI), and intergenic spacer (IGS) regions have been reported (21, 78–81). Many targets are being used for further genotyping directly on DNA from fecal samples or from *Giardia* isolates. SSU rRNA, glutamate dehydrogenase (GDH), TPI, β-giardin, IGS region, and elongation factor 1-alpha (EF1-alpha) gene amplification products are used for direct sequencing or RFLP in post-PCR analysis (82–85). Recently, novel sequence information was used to identify new assemblage A- and B-specific loci. Two assemblage-specific PCRs based on these loci showed an excellent performance when used on DNAs extracted from feces (86, 87). The molecular epidemiology of *Giardia* and genotyping methods have recently been reviewed (88, 89). Genotypes of *Giardia* are traditionally named "assemblages," which are identified by analysis of single or multiple loci such as SSU rDNA, β-giardin, TPI, and GDH; the SSU rRNA and GDH genes appear to be the genes that are most easily amplified (84, 90). Humans host mainly assemblage B, while assemblage A is less common; both assemblages are shared with many other mammals (91). Monis and coworkers have suggested using *G. duodenalis* for assemblage A and renaming assemblage B *Giardia enterica*. Assemblages C and D are found mainly in canids and have been referred to as *Giardia canis*; assemblage E, found in livestock, has been referred to as *G. bovis*; assemblage F, found in cats, has been referred to as *G. cati*, and assemblage G, found in rodents, has been referred to as *G. simondi*, but for assemblage H in marine vertebrates, no species name has yet been proposed (88, 89, 92–95). The zoonotic potential of both assemblages A and B is evident when studied at the levels of assemblages and subassemblages and even at each single locus. However, when genotypes are defined by using a multilocus sequence typing scheme, only 2 of 84 multilocus genotypes (MLGs) of assemblage A and none ($n = 99$) of assemblage B appear to have zoonotic potential (88, 96, 97).

While molecular markers for assemblage A appear to produce robust and easy-to-read sequences, the allelic heterozygosity shown to exist at the single-cell level in assemblage B isolates and sometimes further complicated by other coinfecting assemblage B subgenotypes (91) makes precise identification impossible. Therefore, development of alternative genotyping methods appears to be relevant.

Findings on the clinical significance of different assemblages have been contradictory; assemblage B was recently shown to be associated with flatulence in children, and assemblage B appears to be more common in patients with suspected treatment failure (98). Homan and Mank found that assemblage A isolates were detected solely in patients with intermittent diarrhea, while assemblage B isolates were present in patients with persistent diarrhea (99). In Saudi children, a strong correlation between the presence of assemblage B and symptoms was found, while assemblage A was found mainly in cases of asymptomatic giardiasis (85). In contrast, a study in Australia found assemblage A to be associated with diarrhea, while assemblage B was found mainly in asymptomatic children (100). In a study among Rwandan children, assemblage A was associated with vomiting and abdominal pain (101). There are also examples of studies where clinical differences associated with the two assemblages were not identified (102, 103).

As for other parasites, the introduction of PCR-based diagnostic assays for *Giardia* took place about a decade ago. Until then, state-of-the-art diagnosis included mainly microscopy of fecal concentrates (cysts), permanent staining of fixed fecal smears (trophozoites), and antigen detection by using enzyme-linked immunosorbent assays (ELISAs) or direct fluorescent-antibody (DFA) tests typically integrated into an assay also enabling the detection of *Cryptosporidium*. DNA amplification techniques have excellent sensitivity and specificity compared with microscopy and antigen detection (27, 78, 79, 81, 101, 104–108). *Giardia*-specific DNA detection (including all assemblages) is increasingly being incorporated into multiplex assays, which are listed in Table 2.

Dientamoeba fragilis

Dientamoeba fragilis (Fig. 2C) was first described in 1918 as an amoeba of the intestinal tract of humans (109). Later, by means of antigen and ultrastructural studies and analysis of rRNA, however, the organism was reclassified as a trichomonad flagellate although lacking external flagella (110–112). Since its discovery, the pathogenicity of this organism has remained controversial. Although in recent years, several authors have reported the clinical importance of *D. fragilis* as a cause of gastrointestinal symptoms (113, 114), a consensus on its pathogenicity is lacking, mainly because so many *D. fragilis* infections remain asymptomatic (115–118). *D. fragilis* appears to be extremely common and may have a cosmopolitan distribution, although there are large variations in prevalence. *D. fragilis* has been linked to intestinal symptoms, especially in children (119, 120). Some studies report a higher prevalence in patients with intestinal symptoms than in healthy individuals (121), while others report the reverse situation (122). *Dientamoeba* infections are potentially chronic (123), which is one of the reasons why dientamoebiasis has been speculated to be a neglected differential diagnosis of irritable bowel syndrome (IBS) (124). It was found that metronidazole was capable of eradicating *D. fragilis* in 60% of 25 positive patients fulfilling the Rome III criteria for IBS; however, microbiological and clinical cures were not associated, and the study did not support a hypothesis of a simple association between *D. fragilis* and IBS.

The SSU rRNA gene of *D. fragilis* was amplified and completely sequenced for the first time in 1996 (110). Phylogenetic analysis of housekeeping genes such as the SSU rRNA, EF1-alpha, and actin genes consistently reveals *Histomonas*, a potentially invasive parasite causing blackhead disease in birds, as the closest relative (110, 125). Possibly due to insurmountable methodological challenges, such as obtaining sufficient amounts of DNA from axenic cultures, genomic data from *Dientamoeba* are not yet available, and this precludes studies aiming to predict the existence of virulence factors and other effector proteins.

Although one recent paper describes the existence of a cyst stage of *D. fragilis* (112), the apparent absence of a cyst stage has always meant to date that microscopy of fecal concentrates was not applicable, and traditional parasitological detection of *D. fragilis* relies on morphological detection of trophozoites by light microscopy of fixed and permanently stained fecal smears. The sensitivity of a single examination is not high because the day-to-day variation of *D. fragilis* trophozoites in feces seems to be even more

TABLE 3 Primer and probe sequences for PCR-based assays for detection and molecular characterization of *Dientamoeba fragilis*[a]

Target	Method	Amplicon size (bp)	Primer or probe (sequence)	Reference(s)
Detection				
SSU rDNA	PCR	887	DF400 (5′-TATCGGAGGTGGTAATGACC-3′) DF1250 (5′-CATCTTCCTCCTGCTTAGACG-3′)	130, 459, 460
SSU rDNA	Real-time (TaqMan) PCR	77	DF3 (5′-GTTGAATACGTCCCTGCCCTTT-3′) DF4 (5′-TGATCCAATGATTTCACCGAGTCA-3′) Probe (5′-FAM-CACACCGCCCGTCGCTCCTACCG-TAMRA-3′)	127, 138, 451, 460, 461
rDNA (5.8S)	Real-time (TaqMan) PCR	98	Df-124F (5′-CAACGGATGTCTTGGCTCTTTA-3′) Df-221R (5′-TGCATTCAAAGATCGAACTTATCAC-3′) Df-172revT (5′-FAM-CAATTCTAGCCGCTTAT-MGB-3′)	27, 128, 135, 162, 462
Molecular characterization/ genotyping				
SSU rDNA	PCR-RFLP	887	DF400 (5′-TATCGGAGGTGGTAATGACC-3′) DF1250 (5′-CATCTTCCTCCTGCTTAGACG-3′)	130, 459, 460
ITS1-5.8S-ITS2	PCR-sequencing	≈440	TFR1 (5′-TGCTTCAGTTCAGCGGGTCTTCC-3′) TFR2 (5′-CGGTAGGTGAACCTGCCGTTGG-3′)	133
SSU rDNA	PCR	1,700	TRD5 (5′-GATACTTGGTTGATCCTGCCAAGG-3′) TRD3 (5′-GATCCAACGGCAGGTTCACCTACC-3′)	110, 129–131
SSU rDNA	PCR-RFLP	662	DF1 (5′-CTCATAATCTACTTGGAACCAATT-3′) DF4 (5′-CCCCGATTATTCTCTTTGATATT-3′)	85, 90, 131, 463
SSU rDNA	PCR-HRM	662	DF1 (5′-CTCATAATCTACTTGGAACCAATT-3′) DF4 (5′-CCCCGATTATTCTCTTTGATATT-3′)	85
SSU rDNA	Nested PCR-sequencing	366	DF1 (5′-CTCATAATCTACTTGGAACCAATT-3′) DF4 (5′-CCCCGATTATTCTCTTTGATATT-3′) DF322For (5′-GAGAAGGCGCCTGAGAGATA-3′) DF687Rev (5′-TTCATACTGCGCTAAATCATT-3′)	90
ITS1-5.8S-ITS2	PCR-sequencing	500	ssu2 (5′-GGAATCCCTTGTAAATGCGT-3′) lsu1 (5′-AGTTCAGCGGGTCTTCCTG-3′)	134
ITS1	PCR-sequencing	≈100	ssu2 (5′-GGAATCCCTTGTAAATGCGT-3′) 5.8s1 (5′-TGTGAGGAGCCAAGACATCC-3′)	134
ITS1	Nested PCR-sequencing	366	SSU2 (5′-GGAATCCCTTGTAAATGCGT-3′) Df-ITSRev (5′-GCGGGTCTTCCTATATAAACAAGAACC-3′) Df-ITSnesFor (5′-ATACGTCCCTGCCCTTTGTA-3′) Df-ITSnesRev (5′-GCAATGTGCATTCAAAGATCGAAC-3′)	90
SSU rDNA	PCR-pyrosequencing	129	D.FRAGILISpyroF (5′-CGGAGGTGGTAATGACCAGTTAT-3′) D.FRAGILISpyroR (5′-[biotin-C_6]-TTGCAGAGCTGGAATTACCG-3′) D.FRAGILISpyroS (5′-TGGTAATGACCAGTTATAA-3′)	132
SSU rDNA	PCR-sequencing	364	DFpn_1f (5′-GCCAAGGAAGCACACTATGG-3′) DFpn_364r (5′-GTAAGTTTCGCGCCTGCT-3′)	136
Actin	PCR-sequencing	134–840	DF ACTIN_3f (5′-CCACACATTCTACAACGAATTAC-3′) DF_ACTIN_157f (5′-TTCTTTCACTTTACTCATCAGGTC-3′) DF_ACTIN_291r (5′-GACCAGCAAGGTTGAGTCTC-3′) DF_ACTIN_843r (5′-TGGACCAGCTTCATTGTATTC-3′)	136
EF-1α	PCR-sequencing	99–836	DF_EF_1f (5′-CTCACTTTGGAAGTTCGAATC-3′) DF_EF_265f (5′-TCAAAGGCTCGTTATGATGAAATC-3′) DF_EF_364r (5′-GAAACCTGAGATTGGAACAAAC-3′) DF_EF_836r (5′-CTGTGTGGCAATCGAAAAC-3′)	136

[a] Abbreviations: SSU, small subunit; ITS, internal transcribed spacer; EF-1α, elongation factor 1α; RFLP, restriction fragment length polymorphism; HRM, high-resolution melting; TAMRA, 6-carboxytetramethylrhodamine; FAM, 6-carboxyfluorescein; MGB, minor groove binder.

irregular than that observed in other intestinal protozoan infections such as *G. lamblia* and *E. histolytica* (113, 126).

A number of PCRs have been developed for diagnosis of and research into *Dientamoeba* (Table 3). Conventional PCRs have been used mainly for confirmation of microscopy results and subsequent characterization of the *Dientamoeba* ribosomal genes in human fecal samples. A real-time PCR targeting 77 bp of the SSU rRNA gene was the first molecular assay developed as a screening tool, potentially replacing expensive and time-consuming parasitological diagnosis (127). Interestingly, with a real-time PCR targeting the 5.8S ribosomal gene, which also included an internal process control, it was shown that no reduction in amplification efficiency could be detected when comparing fresh material with material that had been stored unpreserved at 4°C for 8 weeks (128).

RFLP analysis has been used to distinguish between the two genotypes currently known and which differ by 2% across the SSU rRNA gene (129–131). Genotyping has also been performed by analysis of SNPs detected by PCR and pyrosequencing (132). The Bi/PA strain (GenBank accession no. U37461) is commonly acknowledged as a representative of genotype 2. Although there are still few studies of genotypes, genotype 1 appears to account for the vast majority of cases (130, 131, 133). The value of sequencing of the internal transcribed spacer (ITS) region for typing studies of

D. fragilis is limited due to intrastrain genetic heterogeneity (133). A profiling method using the variability within ITS1 of *D. fragilis* was developed by Bart et al. (134) as a means of extracting useful data from sequenced ITS clones, but so far, little is known regarding its applicability and epidemiological relevance.

Studies of other housekeeping genes, such as EF1-alpha and actin, may prove useful in terms of obtaining a higher resolution than can be obtained by studies of SSU rRNA genes alone, as demonstrated for other metamonads (e.g., *Giardia* and *Trichomonas*) (125). Given the high prevalence of the parasite (135) and the recently discovered potential for zoonotic transmission (90), high-resolution markers for distinguishing between strains are warranted. However, a preliminary study of *D. fragilis* in 40 patients revealed that the EF1-alpha and actin genes appear to be remarkably conserved among patient isolates (136), but the clinical and epidemiological utility of multilocus sequencing of housekeeping genes can be fully determined only after comparing *D. fragilis* isolates from symptomatic cases to those from asymptomatic carriers. The clinical significance of the two known genotypes of *D. fragilis* needs further investigation. Is the rarer genotype more virulent than the other, and could this possibly contribute to the differences in clinical perceptions regarding the organism's pathogenicity?

Until a few years ago, humans were the only known hosts of *Dientamoeba*, but recently, the parasite was discovered in nonhuman primates (gorillas) and pigs as well (90, 137, 138). Unfortunately, no comparisons were made between sequences obtained from humans and those obtained from gorillas, while analysis of *Dientamoeba* ribosomal sequences from pigs showed that these sequences were identical to those of genotype 1 commonly found in humans (90).

Microscopic examination of permanent stains of fixed fecal smears is insensitive compared to nucleic acid-based techniques: in a study comparing microscopy and real-time PCR, Bruijnesteijn van Coppenraet et al. showed *D. fragilis* prevalences of 17% and 31%, respectively (27). Reported prevalence figures may reflect differences in diagnostic modalities as well as geographical variation or age variation in study cohorts (135). Whether or not routine detection of *D. fragilis* should be part of an overall parasitological screen for patients suspected of having intestinal parasitic disease is a matter of contentious debate, mainly due to the predicament that guidelines as to when to try and eradicate the parasite are still to be defined. However, the fact that *D. fragilis* detection can now be easily integrated into multiplex nucleic acid-based detection techniques (Table 2) means that it is relatively inexpensive and straightforward to implement *D. fragilis* PCR in a routine diagnostic panel. First and foremost, this can provide accurate data on possible differences in prevalence and infection intensity between symptomatic and asymptomatic carriers, which can also be exploited in randomized controlled treatment studies for evaluation of treatment efficacy. Moreover, positive DNAs can be stored and used for epidemiological analyses of the prevalence and significance of the two genotypes.

APICOMPLEXA

Some apicomplexan parasites belonging to the suborder Eimeriorina can complete their life cycles in the human intestinal tract and hence can be found in human feces; these parasites include *Cryptosporidium* (Cryptosporidiidae), *Cyclospora* (Eimeriidae), *Cystoisospora*, and *Sarcocystis* (Sarcocystidae).

Cryptosporidium

Cryptosporidium has emerged as an important cause of diarrheal illness worldwide, particularly in young children (<5 years old) and immunocompromised patients (139). At least 6,000 *Cryptosporidium*-caused cases of gastroenteritis occur annually in the United Kingdom, where *Cryptosporidium* is the most common protozoan agent involved in acute gastroenteritis (140). Infections in immunocompetent individuals are self-limiting but may last for 1 to 2 weeks; asymptomatic shedding of oocysts may be common (141). Cryptosporidiosis may be chronic and particularly debilitating in patients with T-cell immune deficiencies, with complications such as sclerosing cholangitis and, rarely, biliary cirrhosis and pancreatitis (140).

Transmission is by the fecal-oral route by accidental ingestion of mature oocysts containing infectious sporozoites (140). As the oocysts are immediately infectious, unlike those of *Cyclospora*, infection may result from direct exposure to mammalian (including human) feces, but food and water contaminated by oocysts may also represent a significant vehicle of transmission. Food- and waterborne outbreaks are not uncommon (142) but may be identified only by chance and after ruling out other causes (143).

While treatment options remain limited, nitazoxanide, which is subject to availability and often requires a special license/approval, may reduce the severity of symptoms, which may include watery diarrhea, abdominal cramps, vomiting, mild fever, and loss of appetite (144). Affected children in developing countries may suffer from malnourishment, and in some nonindustrialized countries, cryptosporidiosis may be a significant cause of morbidity and mortality (145, 146). The introduction of highly active antiretroviral treatment (HAART) for immune reconstruction has dramatically reduced the incidence and severity of cryptosporidiosis in patients with HIV/AIDS (140).

In 2010, 6,605 laboratory-confirmed cases of cryptosporidiosis were reported by 21 European Union/European Economic Area countries; however, 4 countries reported zero cases, and 9 countries failed to report (http://www.ecdc.europa.eu/en/publications/Publications/Annual-Epidemiological-Report-2012.pdf). It is likely that in countries where *Cryptosporidium* infections are not notifiable, diagnostic methods are far from standardized. Individual national prevalence estimates can be difficult to obtain, and even when they are available, they should be interpreted carefully due to variability in diagnostic modalities. In other countries, surveillance systems include sub- and genotyping of laboratory-confirmed cases, which is useful for epidemiological and outbreak investigations (147).

The genus *Cryptosporidium* comprises over 20 established species (Table 4), of which the morphologically indistinguishable species *C. parvum* and *C. hominis* (previously *C. parvum* genotype H or genotype 1) account for most human cases (for a list of loci used to discriminate the two species, see reference 148). However, geographical variation may be seen, and both immunocompromised and immunocompetent individuals may be infected by unusual species and genotypes (149). *Cryptosporidium meleagridis*, in particular, appears to be an emerging pathogen and was found at a rate of 12% in a large study of Peruvian HIV-infected cryptosporidiosis patients (150). In some countries, around 10% of all human cryptosporidiosis cases are due to species other than *C. parvum*, *C. hominis*, and *C. meleagridis* (150, 151). *C. parvum* may be seen more commonly in mixed infections in humans than *C.*

TABLE 4 Species and genotypes of *Cryptosporidium* found in humans, listed according to frequency of reporting[a]

Report frequency and species or genotype	Host reservoir(s)	GenBank accession no.				
		Complete SSU rDNA sequence available (~1.75 kbp) (examples)	COWP sequence (~550 bp) (examples)	LIB13	DnaJ-like protein (HSP40)	ITS2
Common						
C. hominis	Humans	AF093489/L16997	GU904404, GU904390, GU904388, GU904389, GQ983374, GQ983372, DQ388389, EU186155	AF190627	AF400132	AF093012
C. parvum	Humans, ruminants	AF093490, AF161856	DQ187314, DQ060433, DQ062120, JX547011, GU904402, GU904400, GU904398	B78618	AF400131	AF093008
C. meleagridis	Birds, mammals (including humans)	AF112574	EU310392, DQ116568, JX568159, GU904403, AB471654, AY166840, AF248742	NA	AF400133	AF381169
Less common						
C. canis	Dog	AF112576	AF266274	NA	NA	NA
C. cuniculus (previously rabbit genotype)	Rabbit	NA	EU437411, GU327782, GU904391, GU904394	NA	NA	NA
C. felis	Cat	AF112575	AF266263	NA	NA	AF093013
C. ubiquitum	Various mammals	AF442484	JX861404, JX861396, JX861405	NA	NA	NA
C. viatorum	Humans	NA	JX984441	NA	NA	NA
Rare						
C. andersoni	Cattle	AB089285, AY954885, AF093496	DQ060431, AB089289, AB514043, AB514044	NA	NA	NA
C. bovis	Cattle	EF514234	NA	NA	NA	NA
C. fayeri	Red kangaroo	AF112570	AF266269	NA	NA	NA
C. muris	Rodents	AF093498	DQ060430, AB089287	NA	NA	AF381167
C. scrofarum	Pig	NA	NA	NA	NA	NA
C. suis	Pig	AF108861	AF266270	NA	NA	NA
C. tyzzeri (previously, mouse genotype I)	Mouse	AF112571	NA	NA	NA	NA
Chipmunk genotype I	Chipmunk, possibly other sciuridae	NA	JX984442	NA	NA	NA
Horse genotype	Horse	NA	EU437416	NA	NA	NA
Monkey genotype	Monkey	AF112569	NA	NA	NA	NA
Skunk genotype	Skunk, possibly other mustelids	NA	NA	NA	NA	NA

[a] Selected information on nucleotide sequences for ribosomal genes (SSU rRNA and ITS2), *Cryptosporidium* oocyst wall protein (COWP), LIB13, and DnaJ-like proteins (or heat shock protein 40 [HSP40]) currently available in GenBank is also shown. LIB13 is a *Cryptosporidium*-specific gene with unknown function. Abbreviations: SSU, small subunit; ITS, internal transcribed spacer; NA, not available.

hominis (152). Human infections due to *C. canis*, *C. cuniculus*, *C. felis*, *C. ubiquitum*, and *C. viatorum*, as well as other species and unusual genotypes, are also emerging (150, 153–155; M. Lebbad, personal communication). It appears that these genotypes are found more frequently in asymptomatic carriers than in patients with symptoms, which suggests that some "unusual" genotypes may be more common than thought (140). The risk of *C. parvum* infections is higher during spring, while *C. hominis* infections peak in late summer and autumn (140). In contrast to human infection by *C. parvum*, infections due to *C. hominis* may result not only in diarrhea but also in nausea, vomiting, malaise, and nonintestinal sequelae (156).

Similar to many other types of intestinal parasitic disease, symptoms due to cryptosporidiosis are nonpathognomonic, and diagnosis should be confirmed by laboratory tests. Cryptosporidiosis should be suspected in any patient with acute gastroenteritis, particularly in young children and if symptoms are prolonged (140). As a consequence, cryptosporidiosis should be a differential diagnosis to other causes of gastroenteritis, including *Giardia*, *Cyclospora*, *Cystoisospora*, microsporidia, noro- and rotaviruses, *Campylobacter*, *Salmonella*, *Shigella*, and enterohemorrhagic *E. coli*, such as *E. coli* O157 (141). However, in many laboratories, *Cryptosporidium* is not traditionally included in test panels for gastroenteritis. Clinical samples appropriate for laboratory diagnosis of cryptosporidiosis were recently reviewed by Davies and Chalmers (140) and include biopsy specimens (jejunal/gastric), bile (obtained by endoscopic retrograde cholangiopancreatography [ERCP]), sputum samples (if respiratory symptoms are present), and antral washouts (in high-risk patients with unexplained sinusitis), in addition to stool samples. Traditional diagnosis relies on microscopy of modified acid-fast-stained fecal concentrates (Fig. 3A) or auramine-phenol staining and/or antigen detection by DFA or immunochromatographic assays.

The vast number of species reported to infect humans makes a genus-specific PCR assay the most appropriate diagnostic approach in routine clinical laboratories. DNA samples can be stored for later epidemiological analysis in research and surveillance laboratories. The main targets for diagnostic PCRs typically

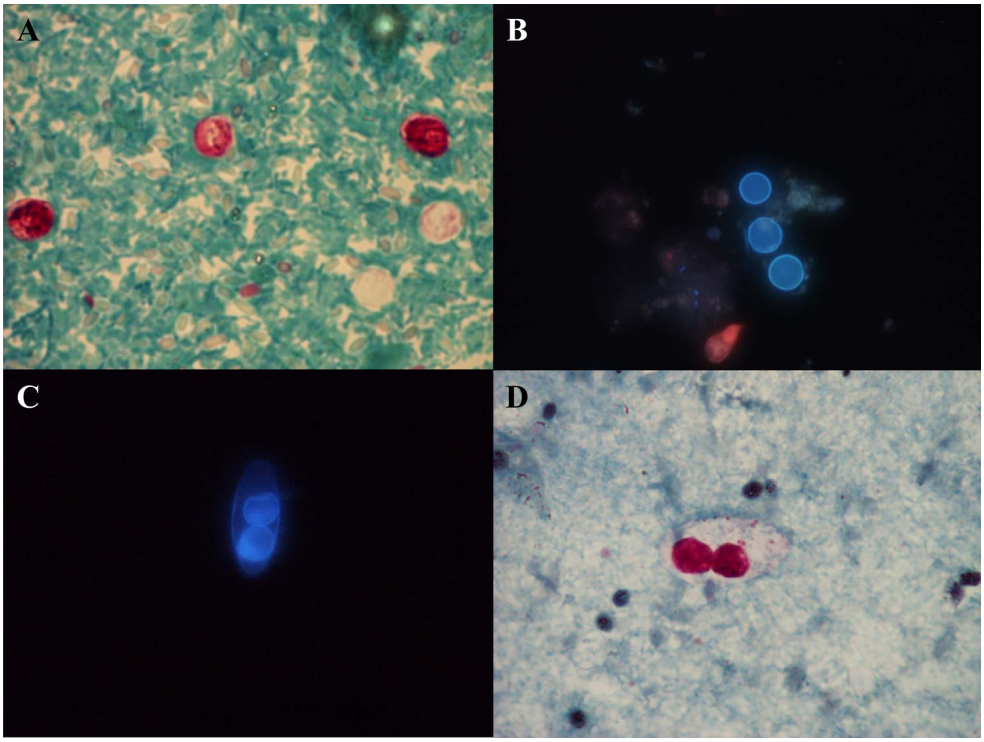

FIG 3 (A and D) Modified Ziehl-Neelsen acid-fast staining of *Cryptosporidium parvum/C. hominis* oocysts (A) and a *Cystoisospora belli* oocyst (D). (B and C) Unstained wet mounts for UV fluorescence microscopy showing autofluorescence of *Cyclospora cayetanensis* oocysts (B) and a *Cystoisospora belli* oocyst (C). (Parasite images courtesy of Marianne Lebbad; reprinted with permission.)

include the SSU rRNA gene, the *Cryptosporidium* oocyst wall protein (COWP) gene, or the DnaJ-like protein gene (157–159). While partial SSU rRNA gene sequences are available for all species of *Cryptosporidium* known to infect humans, surprisingly, only a fraction of these species are currently represented by complete SSU rDNA sequences in GenBank (Table 4). COWP sequences are readily available, but for some other loci, for instance, the DnaJ-like protein used in widely used diagnostic assays (27, 79, 87, 104, 107, 158, 160–162), sequence data are not available for most species other than *C. parvum* and *C. hominis*, and other *Cryptosporidium* species may go undetected by this assay.

Because there is little genetic variation across *Cryptosporidium* SSU rRNA genes, the design of primers/probes targeting the entire genus is relatively straightforward, but there are limited targets for designing species-specific primers/probes. Therefore, other loci have been targeted, such as COWP and the LIB13 locus (a coding region of unknown function) (Table 4), for the differentiation of *Cryptosporidium* species. A few published assays include both genus- and species-specific primers, enabling not only sensitive screening but also real-time identification of at least some species. One such example is a PCR targeting 125 bp of the SSU rRNA gene designed to amplify and partially differentiate *Cryptosporidium* species pathogenic to humans (152). Scorpion probes were designed to enable differentiation between *C. parvum*, *C. meleagridis*, and other species. The assay had a detection limit of 500 to 5,000 oocysts/g feces and was validated against microscopy and antigen detection; species identification by scorpion probes was validated by using RFLP analysis of amplicons, using VspI digestion specific for *C. hominis*. A generic TaqMan assay targeting the SSU rRNA gene and additional TaqMan assays for subsequent distinction between species infecting humans and those infecting cattle were reported recently by Burnet et al. (29).

Some of the challenges associated with developing standardized, "one-size-fits-all," nucleic acid-based tests are exemplified in an interesting paper by Hadfield et al., who introduced a PCR assay based on two duplex reactions. One reaction targeted the entire genus by amplification of the SSU rRNA gene coupled with a reaction targeting the *C. parvum*-specific LIB13 locus (163). The second reaction targeted the *C. hominis*-specific LIB13 locus and also included an internal process control. Hence, the assay allowed direct detection of *C. hominis* and *C. parvum*, and in the event of a positive genus-specific (SSU rDNA) result in the absence of a positive result from either of the two LIB loci, the 300-bp-long SSU rRNA gene product could be sequenced for identification of the species present. It has been argued that SSU rDNA PCR is compromised in its ability to detect mixed species due to preferential amplification of the predominant species in a sample (153, 163, 164). Therefore, the setup developed by Hadfield et al. does not circumvent this potential problem, since this genus-specific PCR is based on SSU rRNA gene amplification. Interestingly, detection and differentiation of *C. hominis*, *C. parvum*, and *C. meleagridis* in human fecal samples can be performed by using high-resolution melting curve analysis of amplicons of the ITS2 region (165). Using this assay, *C. hominis*, *C. parvum*, and *C. meleagridis* were detected in 97, 44, and 2 samples, respectively, of 143 *Cryptosporidium* oocyst DNA samples originating from Australians with clinical cryptosporidiosis, and the results were in agreement with results previously obtained by single-strand conformation polymorphism analysis. Melting curve analyses in assays using intercalating dyes to distinguish between *C. parvum* and *C. hominis*

were also reported by Tanriverdi et al. (166, 167). Fluorescence resonance energy transfer (FRET) probes (Fig. 1B) have also been used to distinguish between *C. parvum* and *C. hominis* (168), but there are issues regarding diagnostic sensitivity and potentially impaired performance in terms of species resolution in mixed infections.

For phylogenetic analysis and molecular epidemiological and outbreak investigations, PCR-RFLP or PCR-sequencing analysis of various loci, including SSU rDNA, COWP, GP60, heat shock protein 70 (HSP70), actin, thrombospondin-related adhesive protein, and many other gene targets, has been useful (169, 170). Although GP60 remains the locus most widely targeted, the ideal combination of loci for molecular epidemiological purposes remains to be identified (169, 171). Surprisingly, there are a number of species and genotypes for which complete SSU rRNA gene sequences are not available (Table 4), including the recently described species *C. cuniculus* and *C. viatorum*. This may be due to the fact that phylogenetic analysis of *Cryptosporidium* isolates are often carried out by using only partial SSU rRNA genes (despite the fact that variation is seen across the entire gene) and sometimes in conjunction with phylogenetic analysis of, for example, HSP70 and actin genes (172, 173).

PCR is slowly gaining a foothold in clinical microbiology laboratories, and the main approach has been the application of generic primers/probes used alone or multiplexed in assays targeting other relevant parasites; typically, these are used as a first-line screening tool as an alternative to traditional diagnostics (Table 2). DNA-based diagnostics offer improved diagnostic sensitivity, as shown by Morgan et al. in 1998 (12) and in a multitude of other studies. One example is seen in a study by Amar et al., who found that PCR resulted in a 22-fold increase in the detection of *Cryptosporidium* and *Giardia* versus conventional microscopy (174). An increased detection rate was found by using a DnaJ-like gene-based TaqMan assay (158) compared to commonly used commercial kits such as Merifluor *Cryptosporidium*/*Giardia* (Meridian Bioscience) and the Immun*Card* STAT! Crypto/*Giardia* Rapid assay (Meridian Bioscience) (175). In a study by Stensvold and Nielsen (162), the multiplex assay was remarkably more sensitive than modified Ziehl-Neelsen staining of fecal concentrates, strongly supporting the implementation of a molecular screening platform for cryptosporidiosis in low-endemicity Denmark: 16/889 samples were positive for *Cryptosporidium* by real-time PCR, compared to none by modified Ziehl-Neelsen microscopy. Along these lines, Chalmers et al. (176) recently showed that the sensitivity of modified Ziehl-Neelsen microscopy was 75.4% compared to the real-time PCR developed by Hadfield et al. (163). Conversely, comparably high sensitivities were reported for auramine-phenol microscopy and commercial kits based on immunofluorescence microscopy and enzyme immunoassays (176). The validation study was carried out by using *Cryptosporidium* samples, 97% of which were either *C. parvum* or *C. hominis*, and therefore, the diagnostic sensitivity reported there must be interpreted in this light.

Cyclospora

Species of *Cyclospora* are obligate intracellular apicomplexan parasites infecting primates and a number of nonprimate hosts, including other mammals, reptiles, and arthropods. *Cyclospora cayetanensis* is the only species so far known to infect humans and is presumably host specific. Oocysts are excreted in feces, and thereafter, it takes >1 week for oocysts to sporulate in the environment. Infection is due to the ingestion of sporulated oocysts. Sporozoites released from ingested oocysts can infect the duodenum and jejunum. Oocysts are highly resistant to disinfectants used in the food industry (177).

Areas where cyclosporiasis is endemic include the Americas, the Middle East, Southeast and South Asia, South Africa, and southern Europe, but the parasite may even be seen in outbreaks in areas where the disease is not endemic, mainly due to the distribution of contaminated food produce such as raspberries, basil, lettuce, sugar snap peas, or other vegetables (178–183) or outbreaks in groups of individuals from areas where disease is not endemic who travel to areas of endemicity (184–186) or as sporadic cases after travel to areas of endemicity (186). Asymptomatic presentation is not uncommon in areas where the disease is endemic (177), but symptoms related to *Cyclospora* infection may include low-grade fever, anorexia, nausea, diarrhea, and weight loss and may be seen primarily in children and HIV/AIDS patients in areas of endemicity (177).

Recently, a study on population-based active surveillance for *Cyclospora* infection by the U.S. Foodborne Disease Active Surveillance Network concluded that clinicians should include *Cyclospora* infection in the differential diagnosis of prolonged or relapsing diarrheal illness and should explicitly request stool examinations for this parasite (187).

Traditional diagnostic methods include mainly autofluorescence (Fig. 3B) or, perhaps more commonly, modified Ziehl-Neelsen staining of fecal concentrates in which oocysts of *Cyclospora* (size, 8 to 10 μm) can be differentiated from those of *Cryptosporidium* (4 to 6 μm) and *Cystoisospora* (25 to 30 μm) by morphological characteristics.

PCR methods in various formats have been used for direct detection (screening) and confirmation of microscopy results. While the ITS region may offer higher resolution and therefore may be better for molecular epidemiological purposes (188, 189), the SSU rRNA gene has been the primary target for PCR-based diagnosis of *Cyclospora* in human fecal samples (Table 5).

In 2003, the first TaqMan assays were reported (190, 191), enabling the incorporation of *Cyclospora* into multiplex screening assays to detect and differentiate multiple genera and species of apicomplexa and microsporidia, with the key driving forces being dramatically increased sensitivity compared to that of microscopy (191, 192) and the fact that these parasites are rarely encountered in clinical samples in areas where the disease is not endemic. A multiplex PCR method to detect *Cyclospora*, *Cystoisospora*, and microsporidia in stool samples based on Luminex technology was recently reported (193). While the probe and reverse primer appear to be genus specific, the forward primer might be species specific for *C. cayetanensis*. The assay could possibly be modified toward the development of a *Cyclospora* genus-specific PCR. It appears relevant to combine diagnostics for intestinal sporozoa (*Cystoisospora*, *Cyclospora*, *Cryptosporidium*, and microsporidia) in multiplex PCR assays, as these parasites are often waterborne, most can be found in outbreaks, and there may be significant overlap in risk factors and symptoms caused by these parasites.

Unfortunately, little has been published on the use of nucleic acid-based tests for the diagnosis of *Cyclospora* infections in a clinical diagnostic setting. In a small study in Peru, nested PCR detected 57.1% positive results in diarrheal cases (20/35) versus only 18.9% positive results in controls (3/15), compared to only

TABLE 5 PCR-based assays for detection and molecular characterization of *Cyclospora*[f]

Species	Amplification method	Detection method	Target	Sample type(s)	Reference
Cyclospora, *Eimeria*	Nested PCR	Gel electrophoresis	SSU rDNA	Purified oocysts	464
Cyclospora, *Eimeria*	Nested PCR[a]	Gel electrophoresis	SSU rDNA	Stool	465
Cyclospora, *Eimeria*	Nested PCR-RFLP[a]	Gel electrophoresis	SSU rDNA	Raspberries	466
Cyclospora, *Eimeria*	Nested PCR[a]	Oligonucleotide ligation	SSU rDNA	Controls	467
Cyclospora, *Eimeria*	Nested PCR[a]	Sequencing	SSU rDNA	Stool	468
Cyclospora	PCR	Gel electrophoresis	ITS1		188
Cyclospora	Real-time PCR	Hydrolysis probe	SSU rDNA	Controls	190
Cyclospora	Real-time PCR	Hydrolysis probe	SSU rDNA	Stool	191
Cyclospora, *Eimeria*	Nested PCR-RFLP	Gel electrophoresis	SSU rDNA	Water	469
C. cayetanensis, *C. colobi*, and *Eimeria*	Multiplex nested PCR[b]	Gel electrophoresis	SSU rDNA	Stool	470
Cyclospora, *Eimeria*	Nested PCR[a]	Gel electrophoresis	SSU rDNA	Sputum	471
Cyclospora, *Eimeria*	Nested PCR[a]	Gel electrophoresis	SSU rDNA	Stool	472
Cyclospora	Real-time PCR[e]	Hydrolysis probe	SSU rDNA	Stool	473
C. cayetanensis, *C. colobi*, and *Eimeria*	Multiplex Nested PCR[d]	Gel electrophoresis	SSU rDNA	Stool	194
Cyclospora, *Eimeria*	Nested PCR-RFLP[a]	Gel electrophoresis	SSU rDNA	Stool	192
C. cayetanensis	PCR	Gel electrophoresis	ITS2	Spiked samples	474
Cyclospora	PCR[c]	Gel electrophoresis	ITS1	Stool	189
	Nested PCR	Gel electrophoresis	SSU rDNA	Controls	450
Cyclospora, *Cryptosporidium*, *Toxoplasma*, and *Sarcocystis*	Real-time PCR	SYBR green MCA	SSU rDNA	Control samples	219
C. cayetanensis, *C. colobi*, and *Eimeria*	Multiplex PCR[d]	Luminex	SSU rDNA	Stool	193
C. cayetanensis, *C. colobi*, and *Eimeria*	Multiplex nested PCR[d]	Gel electrophoresis	SSU rDNA	Stool	184
Cyclospora	Nested PCR	Sequencing	SSU rDNA	Stool	475
Cyclospora	Nested PCR	Sequencing	HSP70	Stool	195
C. cayetanensis, *C. colobi*, and *Eimeria*	Nested PCR[d]	Electrophoresis	SSU rDNA	Raspberry, basil, pesto	476
C. cayetanensis, *C. cercopitheci*	Real-time PCR	Hydrolysis probe	SSU rDNA	Raspberry, basil, pesto	
C. cayetanensis	Real-time PCR	Hydrolysis probe	HSP70	Raspberry, basil, pesto	

[a] PCR primers are described in reference 464.
[b] First-round PCR primers are described in reference 464.
[c] PCR primers are described in reference 188.
[d] PCR primers are described in reference 470.
[e] PCR primers are described in reference 190.
[f] Abbreviations: SSU, small subunit; ITS, internal transcribed spacer; HSP, heat shock protein; RFLP, restriction fragment length polymorphism; MCA, melt curve analysis.

18.9% and 6.7% positive samples in cases and controls, respectively, by microscopy (192). In Egypt, *Cyclospora* infections were detected in 35 of 140 (25%) diarrheic children by using nested PCR, compared to 17.8% and 22.2% with modified Kinyoun stain and autofluorescence microscopy, respectively (194). In The Netherlands, 100 stool specimens from returning travelers with diarrhea were screened for the presence of *C. cayetanensis* by using fluorescence microscopy and real-time PCR. *C. cayetanensis* was found in five cases by PCR, one of which (20%) could be confirmed by microscopy only after examination of several additional slides; *C. cayetanensis* was the most frequent parasitic cause of diarrhea after *G. lamblia* (191).

Genotyping has been performed for the differentiation of *Cyclospora* species by using the ITS region and the SSU rRNA gene. Very few other genes have been explored, and no sequence variation was found in the HSP70 genes of *Cyclospora cayetanensis* isolates from Nepal, Mexico, and Peru (195).

Cystoisospora

The apicomplexan genus *Cystoisospora* (Sarcocystidae; formerly *Isospora*) comprises species that infect humans, dogs, cats, and other mammals (196, 197). While cystoisosporiasis may be common in nonhuman hosts, human infection by *Cystoisospora* appears to be relatively rare, and symptomatic infections are probably seen mostly in immunocompromised individuals, including patients with HIV/AIDS or lymphoproliferative disorders, in whom infections are often chronic and severe (198–201). Superinfection of the small bowel during administration of systemic corticosteroids for eosinophilic gastroenteritis has been documented (202).

Regions of endemicity include tropical Africa, Southeast Asia, and Central and South America; in Western countries, human infections may largely be imported from travel to or origins in countries where the disease is endemic (203).

Infection is probably self-limiting in immunocompetent hosts. Symptoms may include diarrhea, steatorrhea, abdominal pain, fever, malaise, nausea, vomiting, weight loss, dehydration, and cachexia (197). Bile duct infection may be seen, while disseminated disease is probably rare but has been reported for AIDS patients. Recurrences are common, and the chronic nature of the illness contributes to morbidity and mortality among these patients (204–206). Sulfamethoxazole-trimethoprim treatment provides a good clinical response and is the drug of choice (207).

Mature oocysts are infective and contain two sporocysts, each containing four sporozoites. Sporozoites released into the small bowel by ingested oocysts invade the intestinal epithelium, and once intracellular, the parasites undergo schizogony with merozoite, trophozoite, schizont, gametocyte, and oocyst formation (196, 197). Tissue cysts (lamina propria) may be seen by endoscopy in the absence of fecal shedding of oocysts; tissue cysts may be unizoite (208, 209). Oocysts are oblong and characterized by a

thin, transparent shell surrounding the sporocysts and are usually detected in the laboratory by Ziehl-Neelsen staining of fecal concentrates (Fig. 3D) or by phase-contrast or fluorescence microscopy of unstained wet mounts (Fig. 3C). Flotation techniques are sometimes used.

There is some indication that oocyst morphology differs from host to host (210). Very few molecular studies have been undertaken to characterize *Cystoisospora* from humans and other animals, so host specificity and the level of genetic diversity are incompletely known; however, *Cystoisospora belli* (formerly *Isospora belli*) is the species that has been reported in humans.

While Murphy et al. diligently described how *Cystoisospora* can be detected by using a broad-specificity primer approach (211), such an approach is not feasible in general practice and on routine screening platforms due to potential requirements such as cloning and low cost-effectiveness. Reports of targeted PCR assays are scarce but include an assay based on nested PCR (212). This nested PCR used an outer primer pair, IsoFO (5′-GTGCCTCTTCCTCTGGAAGG-3′) and IsoRO (5′-GCACTCCACCCAGTTAAGTGC-3′), and an inner primer pair, IsoFI (5′-CGATGGATCATTCAAGTTTC-3′) and IsoRI (5′-ACCACGTACACACCCCTAAG-3′), followed by probe hybridization (IsoOP [5′-GT{T/A}ATGGCTTCGGCCGGCGATGGA-3′]). While this assay could potentially be implemented in a TaqMan format, the primers have not been validated on fecal DNA. A real-time PCR with internal process control was described by ten Hove et al. (213). It uses primers Ib-40F (5′-ATATTCCCTGCAGCATGTCTGTTT-3′) and Ib-129R (5′-CCACACGCGTATTCCAGAGA-3′) and amplifies an 89-bp fragment of the ITS2 region, which is detected by the double-labeled probe Ib-81Taq (5′-FAM [6-carboxyfluorescein]-CAAGTTCTGCTCACGCGCTTCTGG-BHQ1-3′). The assay was shown to be specific for 147 bacterial, parasitic, and fecal control DNA samples and detected *C. belli*-specific amplification in 21 microscopy-positive stool samples. This assay may be specific for *C. belli*; at least the probe is located in a region where *C. ohioensis* exhibits a vast degree of divergence, including an insertion of 3 bases. *C. ohioensis* is one of several species of *Cystoisospora* identified in synanthropic carnivores (214). However, for several species of *Cystoisospora*, no sequence data are available for this particular region, so the specificity of the probe is difficult to determine at present.

A multiplex PCR with 4 primer sets to amplify *C. cayetanensis*, *C. belli*, *Enterocytozoon bieneusi*, and *Encephalitozoon intestinalis* was developed, in which detection of amplicons occurs by specific probes coupled to Luminex beads (193) (Table 2). For *C. belli*, primers were based on *C. belli* 5.8S rRNA and ITS2 and amplify a 213-bp sequence. These primers may enable the detection of species of *Cystoisospora* other than *C. belli*; currently, only data for *C. ohioensis* are available in GenBank for this region, and only 1 to 2 mismatches are seen across the primers.

The SSU rRNA gene and ITS1 sequences are used to determine the taxonomic status of *Cystoisospora* isolates (210, 214–216).

Sarcocystis

Humans may serve as both intermediate and definitive hosts of *Sarcocystis*; only intestinal *Sarcocystis* infections are briefly discussed here. Although *Sarcocystis* may include distinct species pathogenic to humans, very little is known regarding host specificity, taxonomy, and overall epidemiology (217). Considering the fairly high prevalence in various cohorts across the globe, as reviewed by Fayer (217), it is surprising that reports on *Sarcocystis* infections of humans are so scarce. While DNA-based confirmation is still pending, it is possible that intestinal infection by *Sarcocystis* in humans may be caused by both *Sarcocystis hominis* and *Sarcocystis suihominis* and acquired through the consumption of tissue cysts in undercooked beef and pork, respectively (217).

Symptoms related to intestinal infection include nausea, loss of appetite, vomiting, stomach ache, bloating, diarrhea, dyspnea, and tachycardia; they appear only a few hours after ingestion of tissue cysts and may last for about 36 h. The prepatent period is 5 to 12 days, and patency may last for at least 120 days. Importantly, symptoms overlap those characteristic of acute gastroenteritis (217).

Seminested PCR combined with RFLP analysis has been used to detect and distinguish *S. hominis*, *S. fusiformis*, and *S. cruzi*-like organisms (218). *Sarcocystis cruzi* was included in a real-time PCR assay targeting various coccidia of animal health and zoonotic importance, where organisms were detected and differentiated based on melting curve analysis (219).

CILIATES

One genus of ciliates is known to be able to infect humans: *Balantidium*.

Balantidium

Balantidium has a cosmopolitan distribution and is present generally wherever pigs are present (220). Both *B. coli* and *B. suis* infections of pigs have been reported, but molecular studies still remain to be performed to settle whether *B. coli* and *B. suis* are in fact separate species. Recent data, however, suggest that *B. coli* has low host specificity (221). A study from Denmark revealed a prevalence of 100% in some pig cohorts, but so far, cases of human balantidiosis acquired in Denmark remain to be reported. It has been suggested that *Balantidium* does not readily produce patent infections in humans (220). While asymptomatic infections may be common, dysentery and invasion of the colon have been reported (220, 222). Bowel perforation caused by *B. coli*, leading to severe peritonitis, was described in France (223). Extraintestinal spread to the peritoneal cavity, genitourinary tract, and lungs has been reported (224–227).

Transmission occurs by ingestion of cysts contaminating food or drink. Risk factors include contact with pigs and pig excreta (224, 228). In humans, *B. coli* is the species reported; the same species is known to infect pigs. It is the only ciliate and the largest protozoon known to infect humans, and while human infections may be rare, *Balantidium* may be extremely common in other hosts, including pigs, causing asymptomatic infections. In fact, a number of ciliates are known to colonize ruminants, to which hosts these parasites are often beneficial, assisting the host in food digestion and carbohydrate metabolism (229).

DNA or cysts of *Balantidium* have been detected in fecal samples from diverse hosts such as humans and nonhuman primates, pigs, ostriches, rats, guinea pigs, salamanders, frogs, and fish (230–234) and even in nonfecal human specimens such as bronchoalveolar lavage (BAL) fluid (224–227).

Because the cysts measure 40 to 60 μm and trophozoites might be as large as 200 μm and are easily overstained in iodine-stained wet mounts, they may easily be missed when focusing on finding protozoan cysts by routine microscopy.

So far, PCR has been used mainly to characterize *Balantidium*

from various hosts, including humans (221, 232–234). Very few *Balantidium* sequences are currently available in GenBank, and most represent the SSU rDNA or ITS regions of *B. coli*. Diagnostic PCRs remain to be reported.

Application of general, broad-specificity primers targeting nonhuman eukaryotic SSU rDNA may be of significant utility in efforts to cost-effectively screen for *Balantidium* in clinical samples other than feces (e.g., biopsy specimens and BAL fluid, etc.).

MICROSPORIDIA

Microsporidia are obligate, intracellular, single-celled fungi that infect a wide variety of vertebrate and invertebrate hosts, comprising >160 genera representing over 1,500 species, at least 14 of which are known to be able to infect humans. Intestinal microsporidiosis in humans is due mainly to *Enterocytozoon bieneusi* and *Encephalitozoon* species, mainly *E. intestinalis* and, probably to a lesser extent, *E. cuniculi* and *E. hellem* (235–238). *E. bieneusi* is generally the species associated most frequently with human infections, although a recent study on Russian HIV-positive patients found that *E. intestinalis* was much more common than *E. bieneusi* in this particular cohort (239). In humans, opportunistic infections associated with persistent diarrhea and weight loss may be seen, especially in individuals with HIV/AIDS and organ transplant patients, while in immunocompetent individuals, symptomatic, self-limiting infection may occur (240–245). In HIV-infected patients, a $CD4^+$ T-cell count of <100 cells per μl appears to be a significant risk factor for microsporidiosis (246–248), although a study of HIV patients in Nigeria reported microsporidiosis associated with a $CD4^+$ T-cell count of <200 cells per μl (249); in a study of Danish HIV patients with unexplained diarrhea, most of whom were treated with HAART and had $CD4^+$ T-cell counts of >100 cells/μl, no cases of microsporidiosis were detected by PCR for *Enterocytozoon* and *Encephalitozoon* (160). Infections due to *Encephalitozoon* may also cause rhinosinusitis, keratoconjunctivitis, nephritis, hepatitis, and systemic infections.

Probably because of the increased attention to microsporidiosis during the AIDS epidemic and the improvement of diagnostic techniques, microsporidiosis is now increasingly being diagnosed in transplant patients, children, the elderly, and travelers (241). Although reports of outbreaks of microsporidia are scarce, both food- and waterborne outbreaks have been reported (250, 251). Most infections in immunocompetent individuals are self-limiting; in immunocompromised patients, primary treatment may include chemotherapeutic intervention with the aim of restoring immunocompetence, such as administration of granulocyte colony-stimulating factor in combination with the use of antibiotics such as nitazoxanide, albendazole, or fumagillin (252, 253).

The traditional gold standard for the detection of microsporidia relies on the demonstration of 1- to 2-μm spores by transmission electron microscopy, but this is an insensitive method for finding spores, since the specimen analyzed is relatively small. Detection of microsporidia in stool samples often includes the identification of spores in fecal smears by nonspecific histochemical chemofluorescent agent stains or trichrome stain as well as monoclonal antibody immunofluorescence assays (246, 254–256). Even using these staining techniques, detection and identification of microsporidial spores are difficult, and expertise is required. PCR has been shown to be a valuable technique in the diagnosis of these important opportunistic pathogens (238). Moreover, as distinct treatment options are available for different genera, identification to the genus and species levels is clinically important (252).

Molecular diagnostic tests for the detection of microsporidia have been reviewed extensively (238, 257, 258). PCR diagnosis for *E. bieneusi* was developed as early as 1993 (259), and since then, a variety of conventional PCRs for detection and species identification have been described. Meanwhile, relatively few real-time PCR methods have been reported (Table 6). A PCR followed by detection of *E. bieneusi*, *E. cuniculi*, *E. hellem*, and *E. intestinalis* by microarray has been developed (260). The assay developed by Taniuchi et al. included a multiplex PCR setup along with primers and probes for *Cyclospora* and *Cystoisospora* (193). This appears to be a relevant option for the detection of diarrheagenic parasites that are difficult to detect by conventional methods, particularly in susceptible individuals such as HIV/AIDS patients and recipients of organ transplants.

Based on the ITS nucleotide sequence of *E. bieneusi* recovered from feces of infected humans and animals, *E. bieneusi* comprises a perplexing array of genotypes, several of which have been found in humans. New genotypes keep emerging, and thus, the number of *E. bieneusi* genotypes today may be well over 100 (37, 249, 256, 261, 262). Some of these genotypes have been recognized as being host specific, while others have been found to infect both humans and other animals, supporting the likelihood of zoonotic transmission (235). Interestingly, pigeons appear to constitute a reservoir for species of microsporidia seen in humans; however, little is known about the potential overlap of genotypes between humans and pigeons (263).

Genotype B was the only genotype identified in samples from patients with HIV in Australia and was also the predominant genotype in France and The Netherlands (37, 248, 264). In contrast, a variety of genotypes were identified in HIV-infected individuals in Portugal, Peru, Thailand, Niger, Nigeria, Vietnam, and Gabon and in unselected individuals in Cameroon (247, 249, 265–267). Genotype D was recently reported for two Spanish transplant patients (242); in two other studies of organ transplant recipients in Europe, genotype C was the predominant genotype (37, 264). Genotype C was also found to be responsible for a food-borne outbreak in Sweden (250).

E. cuniculi infection in humans is rarely reported, while birds, canids, rabbits, and rodents may be common hosts (236, 268). At least four genotypes are known (269), among which genotype II has not been detected in humans to date to our knowledge.

While genetic variation in *E. intestinalis* remains to be described, *E. hellem* comprises at least 3 genotypes with some intragenotypic variation (269–274). Interestingly, analysis of four *E. hellem* isolates from humans revealed that the C-terminal regions of the spore wall proteins EhSWP1a and EhSWP1b are polymorphic, which is of interest for epidemiological studies (275).

While microsporidia may not currently qualify as part of a routine test panel for intestinal pathogens, PCR-based detection appears to be relevant in cases of HIV-related diarrhea, diarrhea in patients undergoing organ transplantation, and unexplained diarrhea in otherwise immunocompromised patients. Genotyping has immediate relevance to outbreak investigations but has also proven useful in surveillance studies of microsporidia in humans and other animals to identify transmission patterns and other aspects of epidemiology. It appears that there is a plethora of genotypes for *E. bieneusi*, and while ITS sequence analysis may prove valuable for further exploration of the complex epidemiology of

TABLE 6 PCR-based assays for detection and molecular characterization of microsporidia[f]

Species	Amplification method	Detection method(s)	Target	Sample type(s)	Reference
E. intestinalis, *E. cuniculi*, *E. hellem*	Real-time PCR[e]	Hydrolysis probe	SSU rDNA	Controls	477
E. intestinalis, *Encephalitozoon* species	Real-time PCR	FRET probes and MCA	SSU rDNA	Stool (spiked)	478
E. intestinalis	Real-time PCR	Hydrolysis probe	SSU rDNA	Stool, blood, urine, tissue biopsy specimens, and bronchopulmonary specimens	479
E. bieneusi	Real-time PCR	Hydrolysis probe	SSU rDNA	Stool	480
E. bieneusi, *Encephalitozoon* species	PCR-hybridization	Chemiluminescence	SSU rDNA	Stool, urine (spiked)	481
E. bieneusi	Real-time PCR	Hydrolysis probe	SSU rDNA	Stool	482
E. bieneusi, *E. cuniculi*, *E. hellem*, and *E. intestinalis*	PCR	Microarray	SSU rDNA	Stool	260
E. bieneusi, *E. intestinalis*	Real-time PCR[a,b]	Hydrolysis probe	SSU rDNA	Stool	266
E. bieneusi, *E. intestinalis*	PCR	Gel electrophoresis	SSU rDNA	Stool	483
E. bieneusi	Multiplex real-time PCR	Hydrolysis probe	ITS	Positive stool	457
Encephalitozoon species			SSU rDNA		457
E. bieneusi	PCR	Sequencing	SSU rDNA	Positive stool	37
E. bieneusi, *E. intestinalis*	Multiplex PCR[c]	Luminex	SSU rDNA	Stool	193
E. bieneusi, *Encephalitozoon* species	Real-time PCR	SYBR green and MCA	SSU rDNA	Stool	484
E. bieneusi, *E. intestinalis*	Real-time PCR[b,d,e]	Hydrolysis probe	SSU rDNA	Stool	485

[a] *E. bieneusi* primers and probe are described in reference 482.
[b] *E. intestinalis* primers and probe are described in reference 479.
[c] *E. intestinalis* primers and probe are described in reference 457.
[d] *E. bieneusi* primers and probe are described in reference 480.
[e] Singleplex PCRs in parallel.
[f] Abbreviations: SSU, small subunit; ITS, internal transcribed spacer; HSP, heat shock protein; RFLP, restriction fragment length polymorphism; MCA, melt curve analysis; FRET, fluorescent resonance energy transfer.

microsporidia, it may be relevant to identify alternatives to ITS sequence analysis for a more clinically relevant strain identification approach.

Of note, few diagnostic PCRs have been developed for species that potentially may cause systemic infections, and validation studies on DNA extracted from clinical samples other than stool, such as urine and pulmonary samples, remain limited.

STRAMENOPILES

Stramenopiles encompass a range of very diverse organisms, most of which are free-living. *Blastocystis* is remarkable in that it has adapted to a strictly anaerobic parasitic life-style and is capable of colonizing a vast variety of mammalian, avian, reptilian, amphibian, and arthropod hosts. Only one other stramenopile is known to be able to cause parasitic infections in humans, namely, *Pythium*, which is a rare cause of skin disorders and systemic granulomatous disease. *Proteromonas lacertae*, which is closely related to *Blastocystis*, can be found in reptiles.

Blastocystis

Blastocystis (Fig. 2D) is a common, strictly anaerobic, unicellular intestinal parasitic protist of humans and a wide variety of nonhuman hosts (276–278). Despite the fact that it is one of the most common microbial eukaryotes to colonize the human intestine and its clinical significance is largely unknown, it remains a relatively little-studied parasite. This may be due in part to a variety of unrelated predicaments: the inconspicuousness of the parasite, which makes diagnosis based on morphology difficult; the difficulties associated with isolation in axenic culture; the resilience of infections, which may be chronic and difficult to eradicate; and the frequency with which the parasite presents itself along with other intestinal microbial eukaryotes, such as *Dientamoeba*, making it extremely difficult to know whether potential symptoms are due to *Blastocystis* or another organism. It is not known how to eradicate the parasite, and *Blastocystis* is not even remotely genetically related to other microeukaryotes that colonize or infect the human intestine, such as other protists and yeasts. Although all the stages involved in the life cycle of *Blastocystis* have not been fully clarified, transmission is by the fecal-oral route and possibly mostly involves the accidental ingestion of the cyst stage (278).

While asymptomatic carriage is common, *Blastocystis* has been linked to disease in a variety of case reports (279). The parasite exhibits remarkable genetic diversity, and to date, nine genetically distinct lineages, so-called subtypes (STs) (arguably species), have been found in humans, of which ST1 to ST4 account for >90% of human *Blastocystis* carriage (277). Efforts continue to identify potential associations between subtypes and clinical outcomes of colonization; so far, results are pointing in different directions.

Microscopy of fecal concentrates has low diagnostic sensitivity, which has most likely led to substantial underreporting of the parasite (280–282), whereas the use of permanently stained smears of preserved stool specimens will increase the rate of detection of *Blastocystis* (283). The use of molecular tools in *Blastocystis* research and routine diagnostics has had a crucial impact on our understanding of *Blastocystis* epidemiology and transmission. The first report of a diagnostic PCR for *Blastocystis*, in 2006, was partly inspired by the tendency toward screening of fecal DNAs for

common intestinal protozoa by PCR as a supplement to or even as a substitute for microscopy of fecal concentrates (284). Primer design was based on sequences available in the NCBI database at that particular time. Analysis of newer data leads to the conclusion that this PCR assay may exhibit preferential amplification of some STs over others due to sequence variation in primer annealing sites, which may impair its use in epidemiological studies. Indeed, the extensive intrageneric diversity of *Blastocystis* has made the design of a diagnostic genus-specific PCR applicable to fecal DNA templates challenging.

Three diagnostic real-time PCR assays have been reported. A real-time PCR based on an unknown *Blastocystis* gene using FRET probes was validated against ST1, ST3, and ST4 (285). A SYBR green real-time PCR was designed based on the SSU rRNA gene for the detection of *Blastocystis*-specific DNA and subsequent subtyping by melting curve analysis (286). Low sensitivity can be expected due to the relatively large PCR product (320 to 342 bp, depending on the subtype), and the specificity of the assay is only 95%. The third real-time assay, using a hydrolysis probe based on the SSU rRNA gene, was characterized by 100% specificity (287). The use of real-time PCR in large-scale surveys will assist in identifying whether the development of symptoms is related to infection intensity by simple analysis of threshold cycle (C_T) values for individual samples.

Xenic *in vitro* culture (XIVC) sensitivity ranged between 52 and 79% compared to these real-time PCR assays (286, 287). Previously, XIVC was found to have a sensitivity of 89% compared to conventional PCR (280). Conventional PCR relies on visual evaluation of PCR results, and this PCR was based on primers that amplify a relatively large PCR product (~550 to 585 bp), which was suitable for sequencing and subtype identification but too large to be relevant for diagnostic PCR, especially in situations where fecal DNAs are of suboptimal purity. An estimate of the number of rRNA gene copies in one *Blastocystis* cell is not available, although it may lie somewhere between 20 and 100 (287, 288).

Generally, two methods have been employed for the genetic characterization (subtyping) of *Blastocystis*, namely, barcoding (289) and sequence-tagged-site (STS) PCR (290). These two methods were recently evaluated by Stensvold (291), who concluded that barcoding is the method of choice, enabling the detection of novel subtypes and further scrutiny of genetic diversity, including analysis of SSU rRNA alleles, since the barcode region has been validated as a marker of overall genetic diversity of *Blastocystis* (289, 292).

Barcode primers (RD5/BhRDr) amplify 600 bp of the 5′ end of the SSU rRNA gene, and phylogenetic analysis has demonstrated that this region is a valid surrogate genetic marker for complete SSU rRNA gene sequences and even for markers in the genome of the mitochondrion-like organelle (289, 292). The drawbacks compared to the STS method are that sequencing is needed and that mixed-subtype colonization may be difficult to decipher. On the other hand, barcoding enables a more subtle analysis, namely, SSU rDNA allele analysis. A public database is available (http://pubmlst.org/blastocystis/), which includes a sequence depository for barcode sequences and sequences obtained by multilocus sequence typing (MLST) (see below). It also has a BLAST facility, where individual or bulk fasta files can be uploaded and analyzed for quick identification of subtype number, hence obviating the need for phylogenetic analysis (292, 293). SSU rDNA allele analysis is a useful indicator of intrasubtype genetic variation (292), and to date, >35 SSU rDNA alleles for ST3 have been identified, whereas the numbers of SSU rDNA alleles for ST4 and some other subtypes remain much more limited. However, some of the allelic variation in databases is the result of cloning and sequencing of individual genes from strains rather than sequences based on PCR products produced from the whole genome; intragenomic SSU rDNA polymorphism has been reported (288).

Subtyping has revealed significant differences in *Blastocystis* epidemiology: ST4 appears to be common in Europe, while it is generally rare in most other regions. ST6 and ST7 account for about 20% of the cases in Africa, while only sporadic cases are seen in other regions. Independent data from Denmark (294) and Spain (295) show associations between ST4 and diarrhea, and ST4 is also common in United Kingdom patients suffering from IBS (277). Geographical differences in the distribution of subtypes may hamper attempts to identify subtypes specifically linked to disease.

"Genotype" has been used by some authors interchangeably with "subtype." Presuming that *Blastocystis* subtypes are equivalent to separate species, subtype allele analysis should be regarded as the equivalent of genotyping in other organisms. At this level, there is currently very little information on *Blastocystis*. However, it is likely that the introduction of the SSU rDNA allele database at PubMLST (http://pubmlst.org/blastocystis/) will greatly facilitate studies of both subtypes and SSU rDNA alleles, enabling rapid analysis of fasta files obtained by barcoding (289). Indeed, allele analysis is likely to be an essential tool in future analyses of the potential zoonotic transmission of *Blastocystis*.

MLST analysis of *Blastocystis* is currently based on analysis of loci in the mitochondrion-like genome (292); mitochondrial DNA (mtDNA) is especially useful in MLST analyses due to its haploid structure, hence bypassing problems related to sequence heterozygosity, as seen, for example, in some *Giardia* MLST loci (91). So far, MLST systems are available for ST3 and ST4, and similar assays for ST1 and ST2 will follow. Analysis of 132 ST3 and ST4 isolates from humans and nonhuman primates recently revealed dramatic differences in intrasubtype diversity. No fewer than 58 sequence types (SQTs) were detected among 81 ST3 samples, while only 5 SQTs were found among 50 ST4 samples belonging to the common genotype (215). ST4 samples obtained from Denmark, England, and Nigeria shared the same SQT.

While MLST therefore appears to be a very useful tool for investigating patterns of transmission, at least for ST3, the information obtained by the much simpler SSU rDNA allele analysis can be an extremely cost-effective tool. A recent study of *Blastocystis* in NHPs representing 30 genera showed that NHPs typically host the same subtypes as humans, apart from the fact that ST4 is rare in NHPs while ST5 and ST8 are rare in humans. However, despite ST1 and ST3 being common, it was noted that many of the ST3 alleles found in NHPs are not found in humans, and the same holds true for ST1 (296). A large overlap, however, appears to be present for ST2 SSU rDNA alleles, but only MLST can confirm whether or not NHP ST2 isolates are indeed identical to human ST2 isolates.

Due to limited knowledge of the clinical significance of *Blastocystis*, implementation of molecular diagnostics in the routine clinical setting may be considered premature. However, since large-scale epidemiological studies of different cohorts represent a simple pathway to knowledge in *Blastocystis* re-

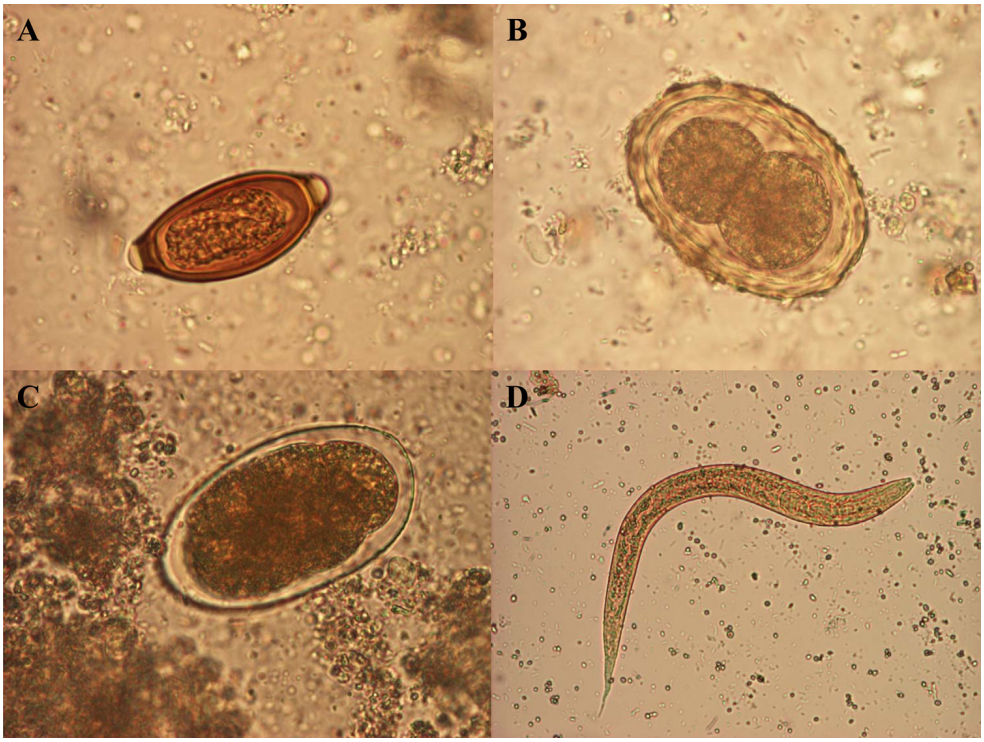

FIG 4 Wet mounts of a *Trichuris trichiura* egg (A), an *Ascaris lumbricoides* egg (B), a hookworm egg (C), and *Strongyloides stercoralis* larva (D). (Parasite images courtesy of Marianne Lebbad; reprinted with permission.)

search, the use of DNA detection methods will most likely prove pivotal in distinguishing between carriers and noncarriers; barcoding can subsequently be used for subtyping and allele identification with no particular *a priori* knowledge by using the sequence query facilities at the PubMLST website (http://pubmlst.org/blastocystis/) (277, 292, 293). In the event that differences in the clinical outcomes of *Blastocystis* infections reflect differences in subtypes or strains, nucleic acid-based methods will most likely be implemented as first-line diagnostics (for recent reviews and updates on *Blastocystis* research, see references 276, 297, and 298).

SOIL-TRANSMITTED HELMINTHS

Eggs of *Trichuris trichiura*, *Ascaris lumbricoides*, *Necator americanus*, and *Ancylostoma duodenale* develop into infective eggs or larvae when excreted onto soil, hence the collective name soil-transmitted helminths (STHs) (Fig. 4). New infections are acquired by ingestion of eggs or penetration of larvae through the skin. With a global estimate that hundreds of millions of people are infected, especially in developing countries, these infections are the most common but also the most neglected infections worldwide (299–302). Although *Strongyloides stercoralis* has a similar infection route, it is usually mentioned only as an aside under the STH heading and is therefore perhaps even more neglected (303, 304), which is strange considering that, as a result of autoinfection, this very chronic disease may suddenly derail, for example, through the use of corticosteroids, leading to a fatal outcome (305–307).

Diagnosis appears to be simple and quite straightforward compared to the difficult microscopic diagnosis of protist infections. However, in Kato-Katz slides, which are often used in epidemiological studies, hookworm eggs will disappear if slides are not read within 30 to 60 min. The diagnosis of *S. stercoralis* infection is notoriously difficult due to the often very small numbers of larvae in chronic infections; multiple stools have to be tested by using Baermann and coproculture techniques to achieve adequate sensitivity (308). Conventional and real-time PCRs for the detection and quantification of soil-transmitted helminths have been developed but are still used mainly for epidemiological studies in areas of endemicity (Table 7).

Trichuris trichiura

To date, only two papers have been published on the use of PCR for the detection of *T. trichiura*-specific DNA with an intended use as a first-line diagnostic tool. This might be due to difficulties in the isolation of parasite DNA from the very robust *Trichuris* eggs (17, 161). The first PCR targeting the *T. trichiura* SSU rRNA gene for detection and quantification of *T. trichiura* infections was incorporated into a real-time PCR in a TaqMan array card format that detected 19 enteropathogens (309). By testing analytical performance using spiked stool samples, the assay showed 100% sensitivity and specificity. Clinical samples from Tanzania and Bangladesh with a variety of known pathogens detected by conventional assays showed *T. trichiura*-specific DNA amplification in 8 of 8 microscopy-positive samples, and an additional infection was detected in 1 of 80 microscopy-negative samples. In a recent paper, a *T. trichiura*-specific singleplex real-time PCR designed from the ITS1 sequence was performed as part of a screen of stool samples collected from schoolchildren in rural Ecuador in parallel with seven additional singleplex PCRs for other intestinal parasitic infections (18). PCR detected *T. trichiura*-specific DNA in 12 of 400 sam-

TABLE 7 PCR-based assays for detection and molecular characterization of intestinal nematodes[h]

Species	Amplification method	Detection method(s)	Target(s)	Sample type(s) (no. of samples)	Reference
O. bifurcum	Nested PCR	Gel electrophoresis	ITS2	Stool (119)	486
N. americanus, *O. bifurcum*	Nested PCR[a]	Gel electrophoresis	ITS2	Stool (262)	13
A. duodenale, *N. americanus*	Nested PCR[b]	Gel electrophoresis	ITS2	Stool (503)	487
Ancylostoma, *N. americanus*, *O. bifurcum*	Multiplex real-time PCR	Hydrolysis probe	ITS2	Stool (339)	328
A. lumbricoides	Nested PCR	Gel electrophoresis and sequencing	Cytb	Coprolites (9)	488
A. lumbricoides	Nested PCR	Gel electrophoresis and sequencing	ITS1, Cytb	Stool (13)	318
S. stercoralis	Real-time PCR	Hydrolysis probe	SSU rDNA	Stool (370)	354
A. duodenale, *N. americanus*, *Trichostrongylus*	Nested PCR	Gel electrophoresis	ITS1-5.8S-ITS2	Stool (203)	489
Trichuris vulpis, *T. trichiura*	Nested PCR	Gel electrophoresis	SSU rDNA	Human stool (80), dog stool (79)	311
Ancylostoma, *N. americanus*, *A. lumbricoides*, *S. stercoralis*	Multiplex real-time PCR[c,d]	Hydrolysis probe	ITS2 (*Ancylostoma*, *N. americanus*), ITS1 (*A. lumbricoides*), SSU rDNA (*S. stercoralis*)	Stool (1,312)	319, 321, 333
Ancylostoma, *N. americanus*, *A. lumbricoides*, *S. stercoralis*	Multiplex real-time PCR[e]	Hydrolysis probe	ITS2 (*Ancylostoma*, *N. americanus*), ITS1 (*A. lumbricoides*), SSU rDNA (*S. stercoralis*)	Stool (78, 229)	161, 320
S. stercoralis	Real-time PCR	Hydrolysis probe	28S	Spiked stool	353
S. stercoralis (and *O. viverrini*)	Real-time multiplex PCR	FRET probes-MCA	SSU rDNA	Stool	356
Ancylostoma, *N. americanus*, *A. lumbricoides*, *S. stercoralis*	Multiplex PCR[e]	Luminex detection	ITS2 (*Ancylostoma*, *N. americanus*), ITS1 (*A. lumbricoides*), SSU rDNA (*S. stercoralis*)	Stool (319)	322
Ancylostoma, *N. americanus*, *A. lumbricoides*, *S. stercoralis*, *T. trichiura*	Real-time PCR[c,d]	Hydrolysis probe	ITS2 (*Ancylostoma*, *N. americanus*), ITS1 (*A. lumbricoides*, *T. trichiura*), SSU rDNA (*S. stercoralis*)	Stool (525)	18
T. trichiura, *A. lumbricoides*	Real-time PCR[f]	Hydrolysis probe	SSU rDNA (*T. trichiura*), ITS1 (*A. lumbricoides*)	Stool	309
N. americanus	Real-time PCR	SYBR green	ITS2	Stool (216)	332
A. duodenale, *N. americanus*	Nested PCR[g]	Gel Electrophoresis	ITS2	Stool (634)	490
N. americanus, *A duodenale*, *A. ceylanicum*, *A. caninum*, *A. braziliense*	Real-time PCR	SYBR green-HRM	ITS2	Stool (634)	330
S. stercoralis	Real-time PCR[d]	Hydrolysis probe	SSU rDNA	Stool (160)	355
Ancylostoma, *N. americanus*	Real-time PCR[c]	Hydrolysis probe	ITS2	Stool (195)	329

[a] *O. bifurcum* primers are described in reference 486.
[b] *N. americanus* PCR primers and probes are described in reference 13.
[c] *A. duodenale* and *N. americanus* PCR primers and probes are described in reference 328.
[d] *S. stercoralis* PCR primers and probe are described in reference 354.
[e] PCR primers and probes are described in reference 319.
[f] *A. lumbricoides* PCR primers are described in reference 319.
[g] PCR primers are described in reference 487.
[h] Abbreviations: SSU, small subunit; ITS, internal transcribed spacer; MCA, melt curve analysis; HRM, high-resolution melt curve analysis; Cytb, cytochrome *b*; FRET, fluorescent resonance energy transfer.

ples, whereas direct wet mount slides identified 3 of 400 samples with *T. trichiura* eggs only. There was a significant correlation between egg counts measured by the Kato-Katz method and the *T. trichiura*-specific DNA load quantified by PCR.

Phylogenetic analysis of *Trichuris* SSU, ITS1, 5.8S, ITS2, and mitochondrial genes derived from worms and eggs isolated from different host species has been performed. Although *T. trichiura* and *T. suis* cannot be differentiated by using morphological and biometric measurements, the ITS1 and ITS2 sequences of *T. trichiura* and *T. suis* isolated from nonhuman primates and different porcine hosts revealed clear differences between the two *Trichuris* species (310). Phylogenetic analysis of the ITS1, ITS2, and SSU rDNA sequences from various *Trichuris* species showed that *T. trichiura* and *T. suis* are closely related but genetically distinct species (310–312). A detailed analysis of the complete mitochondrial genome also confirmed that *T. trichiura* and *T. suis* are separate species (313). Sequence analysis of the ITS2 region of *Trichuris* species collected from pigs and from humans in the Kabale district of Uganda showed that cross-infections of humans with *T. suis* occurs but that cross-infections of pigs with *T. trichiura* were not found. The genetic differentiation and the presence of the two species in humans were confirmed by sequence analysis of a part of the β-tubulin gene. In three cases, worms in humans showed both the *T. suis* and *T. trichiura* ITS2 genotypes, suggesting the existence of heterozygous worms (314). Combining ITS1-5.8S-ITS2 sequences from adult *Trichuris* sp. worms isolated from baboons with GenBank records for *Trichuris* isolated from other humans, other nonhuman primates, and pigs revealed two distinct *Trichuris* genotypes separated from *T. suis* infect-

ing baboons and human patients from Cameroon (315). The zoonotic potential of *T. suis* and different *Trichuris* genotypes is yet not fully understood, and further studies on these sympatric *Trichuris* infections are needed.

Pyrosequencing assays have been developed for the detection of single-nucleotide polymorphisms (SNPs) in the β-tubulin gene that are associated with benzimidazole resistance. The finding of a benzimidazole resistance-associated codon 200 TAC SNP in *T. trichiura* might explain the reported ineffectiveness of benzimidazole anthelmintics against *Trichuris* (316). In Guatemala, by using nested PCR and sequence analysis, nine *T. trichiura*-positive fecal samples revealed the TTC codon in the β-tubulin gene, which is associated with benzimidazole-sensitive parasites (317).

Ascaris lumbricoides

Conventional PCR assays targeting the ITS1 region and the cytochrome *b* gene using gel electrophoresis for the detection of the PCR product were used to detect and genotype *Ascaris* DNA from coprolites found in pre-Columbian South American archaeological sites and in fecal samples from patients attending a health center in Rio de Janeiro, Brazil (318). Another PCR for the detection of *A. lumbricoides* DNA designed from the ITS1 sequence (319) was used in different platforms as part of panels or multiplex assays for the simultaneous detection of several linked pathogens. A multiplex real-time PCR using hydrolysis (TaqMan) probes (18) for the detection and quantification of *A. lumbricoides*, *N. americanus*, *A. duodenale*, and *S. stercoralis* with an internal control was validated against a large panel of control samples and was used in Malaysia and Indonesia (161, 319–321). In two studies in Malaysia, *Ascaris* was detected by PCR in 6/77 and 12/225 cases, respectively, compared to detection in 3/77 and 7/225 cases by microscopy. Microscopy-negative samples showed higher median C_T values (i.e., lower DNA loads) than did microscopy-positive samples. In Indonesia, in a large placebo-controlled study on the effect of albendazole treatment on malarial parasitemia and allergy, *Ascaris* prevalence and DNA load measured by PCR showed little decrease in the placebo group ($n = 466$), and although the decline in the albendazole group ($n = 423$) was much greater, there were still positive cases after treatment 6 times at 3-month intervals. This ITS1-based *Ascaris* PCR was also validated for use in a TaqMan array card for the simultaneous detection of 19 enteropathogens, including viruses, bacteria, protozoa, and helminths (*A. lumbricoides* and *T. trichiura*) (309). The same primer-and-probe design for the detection of *A. lumbricoides* DNA was incorporated into a multiplex PCR for the detection of three intestinal protozoa and four helminths using probe-based detection with Luminex beads (322). TaqMan probe chemistry with another primer-and-probe design from the ITS1 sequence was used in a study in which an *Ascaris* PCR was performed in parallel with singleplex PCRs for seven additional parasitic targets (18). Compared to microscopy, only a small number of additional cases were detected by PCR (28/400 versus 22/400). The microscopy-positive samples showed a much higher DNA load than did the PCR-positive/microscopy-negative samples. DNA loads showed an excellent correlation with the egg counts measured by the Kato-Katz method. In an additional group of 125 children who were tested before and after anthelmintic treatment, all initial *Ascaris* PCR-positive cases tested negative 21 days after treatment.

Whole-genome fingerprinting by amplified fragment length polymorphism (AFLP) analysis, RFLP analysis, and sequencing of the ITS1 and mitochondrial genes and microsatellites have been used as genotyping methods to study the epidemiology and zoonotic potential of *Ascaris*. These studies have been reviewed from different angles to elucidate the taxonomic status of *A. lumbricoides* and *A. suum* and their zoonotic potential (323–325). Several studies using different markers showed zoonotic potential of *Ascaris*, as humans and pigs were found to share common haplotypes. Although some researchers leave the taxonomic status undefined, Leles et al. concluded that based on nematode cross-infections between humans and pigs, the hybridization between *A. lumbricoides* and *A. suum*, and the high levels of genetic similarity between the complete mtDNA genomes, there is a single interbreeding population of *Ascaris*, and those authors recommended synonymizing the two species with the name *A. lumbricoides* Linnaeus 1758 (326).

The codon 200 TAC SNP in the β-tubulin gene was not found in any of the *A. lumbricoides* samples ($n = 38$) tested by using a pyrosequencing assay for the detection of SNPs in this gene that are associated with benzimidazole resistance (316). In Guatemala, sequence analysis of the β-tubulin gene of microscopy-positive *A. lumbricoides* samples showed the TCC codon that is associated with benzimidazole-sensitive parasites in all samples (317).

Hookworm

As reviewed by Gasser et al., species-specific markers defined in the ribosomal and mitochondrial genes were validated for species identification of adult hookworms and were later employed in epidemiological studies using conventional PCR for the detection of species-specific hookworm DNA in fecal samples (327). Primers and probes designed from the ITS2 sequences of *N. americanus* and *A. duodenale* showed high specificity (100%) and sensitivity (98.5% to 100%) using a range of controls and field samples from northern Ghana in a multiplex real-time PCR for the detection of *N. americanus*, *Ancylostoma*, *Oesophagostomum bifurcum*, and an inhibition control (328). Moreover, there was a good correlation between egg counts and parasite-specific DNA load. In an area of low *N. americanus* endemicity in southern Ghana, the detection rate by PCR for a single fecal sample almost equaled that of Kato examination of three fecal samples. Additionally, *N. americanus* infections were detected in 8 of 31 microscopy-negative cases by PCR using three consecutive fecal samples (329). The same primer-and-probe design from the ITS2 sequence was used in multiplex TaqMan-based assays targeting different helminth infections, in singleplex assays tested in parallel with other targets, and in a multiplex PCR combined with probe-based detection with Luminex beads (Table 7). Considerable numbers of additional *N. americanus* and *Ancylostoma* cases were detected with a helminth pentaplex real-time PCR targeting the ITS2 sequences of *N. americanus* and *A. duodenale* in microscopy-negative fecal samples in two studies in Malaysia (161, 320). *N. americanus* DNA was detected in 21/76 and 20/225 microscopy-negative samples, and *Ancylostoma* DNA was detected in 11/76 and 14/225 samples. Because of the identity between ITS2 sequences of the different *Ancylostoma* species, the exact identification of the *Ancylostoma* species involved in these studies is as yet unclear. Real-time PCR targeting the ITS2 sequence followed by HRM analysis enabled the detection and differentiation of *N. americanus*, *A. duodenale*, *A. ceylanicum*, and *A. caninum* (330). Moreover, this PCR assay was more sensitive for the detection of *N. americanus* than was nested

PCR for testing of 634 samples from Malaysia. In a study in Cambodia, microscopy was compared with these *N. americanus* and *Ancylostoma* PCRs in a singleplex format. Although hookworm-specific amplification was detected in an additional 35 of 166 microscopy-negative fecal samples, both PCRs remained negative for 11 of 52 samples in which hookworm-like eggs were seen by microscopy. Moreover, there was no difference in the PCR C_T values (or DNA load) detected in microscopy-negative and microscopy-positive samples (331). The possibility of a less optimal DNA isolation procedure or the misidentification of hookworm-like eggs (e.g., *Trichostrongylus*) can therefore not be excluded. A SYBR green-based real-time PCR targeting the ITS2 sequence of *N. americanus* detected one egg in 200 mg of fecal material and detected *N. americanus*-specific DNA amplification in stool samples from 49 of 261 subjects, compared to 30 cases detected by microscopy (332). PCR-based detection showed large reductions in the percentage of *N. americanus*-positive subjects as well as in the intensity, measured as *N. americanus*-specific DNA loads, in stool samples following intensive albendazole treatment compared to placebo in a study in Indonesia (333). Unexpectedly, PCR revealed that *A. duodenale* and not *N. americanus* was the most prevalent hookworm infection, and *A. duodenale* was shown to be a key factor in severe anemia and iron deficiency in Malawian children (334). Infections with moderate and high *A. duodenale* DNA loads were positively associated with severe anemia, with odds ratios (ORs) of 2.49 (95% confidence interval [CI], 1.16 to 5.33) and 9.04 (95% CI, 2.52 to 32.47), respectively. Iron deficiency measured in bone marrow was positively associated with an increasing *A. duodenale* DNA load (ORs of 3.63 [95% CI, 1.18 to 11.20], 16.98 [95% CI, 3.88 to 74.35], and 44.91 [95% CI, 5.23 to 385.77] for low, moderate, and high loads, respectively).

Genetic diversity within hookworm species investigated by whole-genome fingerprinting methods such as random amplified polymorphic DNA (RAPD) and AFLP analyses, different mutation scanning methods (e.g., SSCP), and sequencing of selected protein-coding mitochondrial genes was reviewed by Gasser et al. (327, 335). Population substructuring was revealed within *A. duodenale* in China by using PCR-SSCP to detect genetic diversity in the *cox1* gene (336). Using adult worms from Africa, Asia, and South America, multiple genetically distinct groups of *N. americanus* were shown by using AFLP (337), confirming previous findings of ribosomal and mitochondrial genetic variation (327).

Single-nucleotide polymorphisms in the β-tubulin gene at codons 167 and 200 have been associated with resistance to benzimidazole anthelmintics in several strongylid nematodes of livestock. A real-time PCR for the detection of these polymorphisms in *A. caninum*, *A. duodenale*, and *N. americanus* was performed on hookworm specimens from schoolchildren in Uganda for whom a reduced response to treatment with mebendazole was observed. However, polymorphisms in codons 167 and 200 of the β-tubulin gene were not detected in any of these samples (338). Amplification and sequencing primers for pyrosequencing were designed to detect and identify benzimidazole resistance-associated SNPs in the β-tubulin genes of *N. americanus*, *A. lumbricoides*, and *T. trichiura* (339). Recently, new-generation sequencing and analysis of the *N. americanus* transcriptome revealed 18 predicted drug targets, which did not have homologues in the human host (340).

Strongyloides stercoralis

Strongyloides stercoralis is endemic to many tropical and subtropical regions; it is estimated that 30 million to 100 million people worldwide are infected, with prevalence rates in some cohorts being as high as 50% (299, 303, 341). *S. stercoralis* behaves differently than other intestinal nematodes; rhabditiform larvae are excreted in the stool but can already develop into infective filariform larvae in the gut and then penetrate the intestinal mucosa or perianal skin, resulting in a prolonged cycle of autoinfection, which can continue for many decades (342–344). Chronic strongyloidiasis may result in hyperinfection or systemic strongyloidiasis and is often seen in immunocompromised hosts such as transplant patients, patients receiving chemotherapy, and patients treated with corticosteroids (303, 305, 345–347).

Laboratory diagnosis is based mainly on serology and the detection of *S. stercoralis* larvae by microscopic examination of fecal samples. Microscopy is labor-intensive and, especially in chronic infections, the sensitivity is low due to the often very small number of larvae, and even after formalin-ether concentration, the use of the Baermann method, or coproculture, multiple samples have to be examined to achieve ample sensitivity (348–352).

Recently, a *S. stercoralis* real-time PCR with primers and FRET probes designed from the 28S gene was described and showed high specificity and specificity using controls and spiked samples, but it was not evaluated further on clinical samples (353). Another real-time PCR with primers and a hydrolysis probe based on the SSU rRNA gene sequence of *S. stercoralis* was 10 to 100 times more sensitive than assays designed from *cox1* and a *S. stercoralis*-specific sequence. The assay showed 100% specificity when used on a large panel of DNAs from fecal samples and controls. When applied to stool samples ($n = 212$) from northern Ghana, the sensitivity of the SSU rRNA PCR compared to the Baermann test or coproculture was 86% or 91%, respectively. In samples with positive Baermann and coproculture results, PCR showed 100% sensitivity, but lower sensitivity was achieved in samples with discrepant results by the Baermann and culture methods (354). A lower sensitivity of PCR for patients with lower larval counts was also found in a study in Bangladesh using the same PCR method. A positive PCR result was found in all Harada-Mori culture-positive samples with high and moderate larval loads, whereas only 15% of the samples with a low larval load were found to be positive by PCR (355). The PCR design was evaluated in asymptomatic schoolchildren in Cambodia ($n = 218$) and showed 88.9% sensitivity and 92.7% specificity compared to the combination of the Baermann method and Koga agar culture as the gold standard; a lower sensitivity was found for samples with discrepant results by conventional methods. Additional PCR-positive samples, however, were found in Baermann- and Koga agar-negative samples as well (331). The SSU rDNA primer-and-probe set (354) has been incorporated into multiplex assays on a variety of different platforms (18, 309, 319, 320, 322) (Table 2). In two studies in Malaysia, *S. stercoralis* DNA was detected in 21/76 and 28/225 microscopy-negative samples (161, 320). The SSU rRNA gene was also used as a target in a duplex real-time FRET PCR combined with melting-curve analysis for the simultaneous detection of *Opisthorchis viverrini* and *S. stercoralis* (356) and showed 100% sensitivity and specificity compared to the agar plate culture method, detecting *S. stercoralis* in 32 of 66 stool samples and 30 controls (356). A conventional PCR designed from the 5.8S rRNA gene

amplified *S. stercoralis*-specific DNA in all 16 agar plate-positive samples and in 5 of 30 microscopy-negative samples in a survey of 782 individuals in Iran (357).

An early study using a PCR-RFLP approach targeting the ITS and a partial 23S rRNA gene using larval DNA revealed species-specific amplicon sizes for *S. stercoralis*, *S. fuelleborni*, and *S. ratti*. RFLP showed identical patterns for human isolates of *S. stercoralis* from different parts of the world and differentiated these from a dog isolate of *S. stercoralis* (358). Sequencing of the SSU rRNA gene of larvae isolated from fecal material collected from orangutans and a human working with orangutans identified *S. stercoralis* and *S. fuelleborni*. The ITS1 sequences of 17 *S. fuelleborni* isolates showed high variability, falling into two clusters, without differentiating the orangutan and human isolates (359).

Considering the rather laborious and time-consuming concentration and stool culture methods that have to be applied on multiple fresh stool samples to achieve ample sensitivity in the diagnosis of *S. stercoralis* infections, which are relatively rare in the Western clinical setting, molecular diagnostics appear to be a worthwhile alternative screening method. In a study using stool samples from returning travelers ($n = 2{,}591$), real-time *S. stercoralis* SSU rDNA PCR detected 21 positive cases, compared to only 3 cases detected by using microscopy of Baermann sediments (107). The higher sensitivity of the PCR could be explained by the detection of DNA from larvae that died before arrival of the samples by regular mail, whereas Baermann tests or coprocultures are dependent on the presence of live larvae. In a small study in Argentina, using 17 positive stool samples from strongyloidiasis patients that were confirmed by agar plate culture of the first, second, or third additional sample collected at 1-week intervals, a PCR using the same primers and a PCR with a novel primer set designed from the SSU rRNA gene performed on the first sample showed positive results in all 17 cases (360).

FOOD-BORNE TREMATODES

Food-borne trematode infections are zoonotic infections and are caused by >80 different trematode species (361). The species considered of public health importance are the liver flukes *Clonorchis sinensis*, *Opisthorchis* spp., and *Fasciola* spp.; the lung flukes *Paragonimus* spp.; and a number of intestinal flukes. The small liver flukes *C. sinensis*, *O. viverrini*, and *O. felineus* are endemic to East and Southeast Asia and central northern Eurasia, and western Eurasia (362). Increasing inland fish production causes a local increase in the number of food-borne trematode infections, and increasing international travel, human migration, food trade, and changing eating habits may cause an increase in the number of cases diagnosed in countries where the disease is not endemic (363–365). Infections with liver and lung flukes are rarely fatal, but the burden of disease is considerable, and moreover, *C. sinensis* and *O. viverrini* infections may lead to cholangiocarcinoma (366, 367). Although *Fasciola* spp., *Paragonimus* spp., and intestinal flukes are found worldwide, higher rates of transmission are known for certain areas, such as the Andean region, Africa, and Asia (366).

Detection and differential diagnosis based on microscopic detection of eggs in feces and sputum (only for lung flukes) are notoriously difficult because eggs of different species resemble each other and are often present in very small numbers (362, 366). DNA-based methods for the detection and differentiation of food-borne trematodes have been used, including PCR, nested PCR, real-time PCR, and LAMP, designed based on the *O. viverrini*-specific repetitive DNA fragment, mitochondrial DNA, ITS1, ITS2, and the SSU rRNA gene (Table 8). A real-time PCR with primers and a hydrolysis probe based on the *O. viverrini*-specific repetitive DNA fragment showed positive results in liver tissue samples from Thai patients with hepatocellular carcinoma and cholangiocarcinoma. *O. viverrini*-specific DNA was not detected in 7 control liver specimens from nonprimary liver cancers (368). The ITS2 sequence of *C. sinensis* was used to develop a TaqMan real-time PCR to detect *C. sinensis*-specific DNA in fecal samples. Beside cross-reacting with *O. felineus*, the assay showed high specificity and 100% sensitivity using samples ($n = 74$) with >100 eggs per gram of feces (epg) and 91.4% sensitivity using samples ($n = 70$) with ≤100 epg. Three additional positive samples were detected out of 26 samples in which no eggs were found by microscopy. The C_T values were strongly correlated with the intensity of infections, as determined by egg counts in Kato-Katz smears (369). A similar approach with primers and a hydrolysis probe designed from the *C. sinensis* ITS1 sequence showed specific (with the exception of *O. viverrini*) and sensitive detection of *C. sinensis* in a small number of fecal samples and fish tissue samples (370). A multiplex real-time PCR based on the 2 DNA sequences of *C. sinensis* and *O. viverrini* was developed by using FRET probes and melting curve analysis. *C. sinensis*-specific DNA and *O. viverrini*-specific DNA were amplified and detected in 8 and 30 *C. sinensis*- and *O. viverrini*-positive stool samples, respectively. Surprisingly, no correlation was found between the C_T values and the intensity of infection determined by *C. sinensis* and *O. viverrini* egg counts in stool samples (371). An interesting approach, using MLPA, was used for the identification of *C. sinensis*, *O. viverrini*, and *O. felineus* and differentiation from other closely related species with three probe pairs derived from the ITS1 sequence. The assay was validated by testing DNA samples from adult *C. sinensis* flukes and fecal samples from *C. sinensis*-infected rats (372). The performance of this technique has not yet been validated on clinical samples from areas where *C. sinensis*, *O. viverrini*, and *O. felineus* are endemic.

Primer sets for the detection of *O. viverrini* DNA in stool samples using LAMP were designed from the mitochondrial *nad1* sequence and from the ribosomal ITS1 sequence of *O. viverrini* (373, 374). The ITS1-based LAMP assay showed a 100% sensitivity compared to microscopy when tested on stool samples ($n = 50$) from schoolchildren from an area of endemicity in Thailand, compared to a remarkably low sensitivity of only 24% using conventional PCR on the same target (373).

The phylogenetic relationship between *C. sinensis* and *Opisthorchis* is not yet clear. There are only small differences between *C. sinensis* and *Opisthorchis* across the SSU rDNA, ITS2, and 28S sequences. Based on the ITS1 sequence, *O. viverrini* and *O. felineus* group together and are closely related to *C. sinensis*, whereas analysis of ITS2 and *cox1* sequences suggests that *O. felineus* is more closely related to *C. sinensis* than to *O. viverrini*. In contrast, the ninth intron of the paramyosin gene (Pm-int9), which is more variable than nuclear markers, showed a closer relationship between *O. viverrini* and *C. sinensis* (375, 376). Microsatellite DNA analysis of *O. viverrini* in Thailand using 12 microsatellite loci revealed the existence of genetic diversity and population substructuring in *O. viverrini*, which can be a useful tool to study transmission dynamics and control (377, 378).

Molecular tools for the detection, differentiation, and genetic

TABLE 8 PCR-based assays for detection and molecular characterization of food-borne trematodes[c]

Species	Amplification method	Detection method(s)	Target	Sample type(s)	Reference
O. viverrini	PCR	Gel electrophoresis	*O. viverrini*-specific repetitive DNA fragment	Stool	491
O. viverrini	PCR[a]	Gel electrophoresis	*O. viverrini*-specific repetitive DNA fragment	Stool	492
O. viverrini, *C. sinensis*	Multiplex PCR	Gel electrophoresis	Mitochondrial DNA	Worms, metacercariae, eggs	493
Opisthorchiidae	PCR	Gel electrophoresis	ITS2	Stool	494
O. viverrini	PCR[a]	Gel electrophoresis	*O. viverrini*-specific repetitive DNA fragment	Stool	495
O. viverrini	Real-time PCR	Hydrolysis probe	*O. viverrini*-specific repetitive DNA fragment	Tumor tissue	368
O. viverrini	PCR	Gel electrophoresis	*O. viverrini* genomic DNA clone	Stool	496
O. viverrini, *C. sinensis*, *Haplorchis taichui*	PCR-RFLP	Gel electrophoresis	ITS2	Stool	497
O. viverrini, *H. taichui*	Nested PCR	Gel electrophoresis	*cox1*	Stool	498
C. sinensis, *O. felineus*, *O. viverrini*	Real-time PCR	Hydrolysis probe	ITS2	Stool	369
O. viverrini	Real-time PCR	FRET probes-MCA	*O. viverrini*-specific repetitive DNA fragment	Stool	499
O. viverrini, *H. taichui*	PCR	Gel electrophoresis	ITS1	Stool	489
O. viverrini, *H. taichui*	PCR	Gel electrophoresis	ITS2	Stool	
C. sinensis, *O. viverrini*, *O. felineus*	MLPA	Gel electrophoresis, capillary sequencer	ITS1	Worm	372
O. viverrini (and *S. stercoralis*)	Real-time multiplex PCR[b]	FRET probes-MCA	*O. viverrini*-specific repetitive DNA fragment	Stool	356
O. viverrini, *H. taichui*	HAT-RAPD PCR	Gel electrophoresis	Genomic	Microscopy-positive stool	500
O. viverrini	LAMP	Visual	*nad1*	Stool, fish	374
O. viverrini	LAMP	Real time, visual	ITS1	Stool	373
O. viverrini, *C. sinensis*	Real-time multiplex PCR[b]	FRET probes-MCA	*nad2*	Stool	371
C. sinensis	Real-time PCR	Hydrolysis probe	ITS1	Stool, fish	370
F. hepatica, *F. gigantica*, intermediate *Fasciola*	Sequence-related amplified polymorphism	Gel electrophoresis	Random nucleotide sequence	Worms	501
F. hepatica, *F. gigantica*	PCR-RFLP	Gel electrophoresis	SSU rDNA	Worms	502
F. hepatica, *F. gigantica*, intermediate *Fasciola*	PCR-RFLP	Gel electrophoresis	ITS2	Worms	503
F. hepatica, *F. gigantica*, intermediate *Fasciola*	PCR	Gel electrophoresis	ITS2	Worms	504
F. hepatica, *F. gigantica*	LAMP	Visual	IGS	Worms, cercariae, eggs	505
F. hepatica, *F. gigantica*	Multiplex PCR	Gel electrophoresis	Mitochondrial DNA	Worms, miracidia, eggs, stools	506
Paragonimus	PCR sequencing	Sequence	ITS2	Eggs	507
P. westermani	LAMP	Visual	ITS2	Sputum, pleural fluid, crabs, crayfish	380
P. westermani, *Fasciolopsis buski*, *F. gigantica*	PCR	Gel electrophoresis	ITS2	Worms	508
Paragonimus heterotremus, *P. westermani*, *P. macrorchis*, *P. siamensis*, *P. harinasutai*, and *P. bangkokensis*	Real-time PCR	FRET probes and MCA	ITS2	Feces of experimentally infected cats	509
Paragonimus bangkokensis, *P. harinasutai*, *P. heterotremus*, *P. macrorchis*, *P. siamensis*, *P. westermani*	PCR pyrosequencing	Sequence	ITS2	Metacercariae	510

[a] PCR primers are described in reference 491.

[b] PCR primers are described in reference 499.

[c] Abbreviations: RFLP, restriction fragment length polymorphism; MCA, melt curve analysis; HRM, high-resolution melt curve analysis; SSU, small subunit; ITS, internal transcribed spacer; HSP, heat shock protein; IGS, intergenic spacer region; *cox1*, mitochondrial cytochrome *c* oxidase subunit I gene; *nad1*, NADH dehydrogenase subunit 1; *nad2*, NADH dehydrogenase subunit 2; MLPA, multiple ligation-dependent probe amplification; HAT-RAPD, high-annealing-temperature random amplified polymorphic DNA; LAMP, loop-mediated isothermal amplification; FRET, fluorescence resonance energy transfer.

characterization of *Fasciola* spp. have been reviewed recently (379). Molecular approaches based on ITS1, ITS2, *nad1*, and *cox1* have been used mainly for the differentiation of adult flukes of *F. hepatica*, *F. gigantica*, and the novel "intermediate *Fasciola*" (i.e., hybrid form between *F. hepatica* and *F. gigantica*) and to detect genetic variation among *Fasciola* spp. The assays have not yet been fully validated in population-based studies or for patient diagnosis. DNA-based methods are not widely used for the diagnosis of paragonimiasis in human cases; PCR and PCR followed by (pyro)sequencing, usually targeting the ITS2 sequence, are

used primarily for the detection and identification of *Paragonimus* species from metacercariae, crabs, and experimentally infected animals or for confirmation of eggs found in sputum or stool samples (Table 8). A LAMP assay using primers targeting the ITS2 sequence detected *Paragonimus westermani*-specific DNA in 17 microscopy-positive pleura and sputum samples, and no *P. westermani*-specific DNA was detected in the negative-control sputum samples (380).

Recent outbreaks of opisthorchiasis after eating raw fillets of tench (*Tinca tinca*) in Italy, with cases found in Italy, Austria, and The Netherlands (381), are only one example that urges the need for specific and sensitive diagnostic tools in a clinical diagnostic setting. In such cases, usually only very small numbers of the very small eggs are present, which makes molecular diagnostic tools such as real-time PCR a very appealing and worthwhile approach to confirm the clinical diagnosis.

SCHISTOSOMA

Although adult schistosome worms reside, depending on the species, in the blood vessels around the intestine or urinary bladder and are therefore strictly blood parasites, the eggs of *Schistosoma mansoni* are excreted in feces through the intestinal wall, and the eggs of *Schistosoma haematobium* pass through the bladder wall into the urine. The number of eggs, especially for travelers, is often very small, and therefore, large quantities of feces and/or urine are concentrated to improve the sensitivity of microscopic examination. Due to the often very light infections in settings where the disease is not endemic, serology is much more sensitive than microscopy for the diagnosis of schistosomiasis in travelers (382). Recent studies have shown that the detection of *Schistosoma*-specific DNA can achieve a higher sensitivity for the diagnosis of acute schistosomiasis than serology (383, 384).

The *S. mansoni* and *S. haematobium* tandem-repeat sequences described by Hamburger et al. (385, 386) have been used in a large number of studies using conventional and real-time PCR for the detection of *Schistosoma*-specific DNA in stool, urine, and serum samples as well as the SSU rDNA, 28S, and ITS sequences and mitochondrial genes (Table 9). In areas of endemicity, *Schistosoma*-specific PCR performed on DNA isolated from a small amount of urine or feces has been shown to be more sensitive than microscopic examination (387). In an area of low endemicity in Brazil ($n = 149$), the detection rate of a conventional PCR designed from the *S. mansoni* 121-bp tandem-repeat sequence and performed on DNA isolated from one stool sample (38.1%) was higher than the detection rate found after microscopic examination of Kato-Katz thick smears of three stool samples (30.9%) (388). Although the sensitivity of a genus-specific real-time PCR targeting the *Schistosoma* ITS2 sequence using DNA isolated from 200 μl of urine from schoolchildren in Ghana with ≤50 eggs/10 ml was only 85.2%, the overall detection rate found for PCR was 20.8%, compared to 15.4% for microscopy with 10 ml urine. The higher sensitivity of the real-time PCR resulted in much higher negative predictive values than for microscopy (389). A higher sensitivity of PCR in samples with low egg counts might be achieved by the use of larger volumes of urine samples and filter-based DNA isolation methods (390–393). Latent class analysis of hematuria, microscopy, and PCR targeting the *S. haematobium*-specific DraI repeat sequence using DNA isolated from one-quarter of a filter paper after filtration of 50 ml of urine showed sensitivities of 87%, 70%, and 100%, respectively (392). ten Hove et al. performed a multiplex real-time PCR targeting the *cox1* genes of *S. mansoni* and *S. haematobium* on a selection of duplicate stool samples from 88 subjects in northern Senegal. *S. mansoni*-specific DNA and *S. haematobium*-specific DNA were amplified, and DNA loads showed a significant correlation with microscopic egg counts for *S. mansoni* in stool and *S. haematobium* in urine; however, the sensitivity of PCR was slightly lower than that of microscopy (394). Real-time PCR targeting the *Schistosoma* ITS2 sequence performed on vaginal lavage samples proved to be a promising tool in the notoriously difficult diagnosis of genital schistosomiasis (395, 396). *S. haematobium* DNA in vaginal lavage samples was highly associated with genital mucosal manifestations typical of female genital schistosomiasis (395).

Recently, there has been increased interest in the detection of *Schistosoma* DNA in serum samples for the (early) diagnosis of schistosomiasis. Already in 2002, amplification of the *S. mansoni* tandem-repeat sequence using conventional PCR was shown in sera from two schistosomiasis patients (387). High sensitivities for the detection of *Schistosoma* DNA using a real-time PCR targeting the *S. mansoni* tandem-repeat sequence have been reported, using DNA isolated from 10 ml plasma from patients with chronic disease infected with *S. mansoni*, *S. haematobium*, and *S. japonicum* and from patients with Katayama syndrome. Amplification of the *S. mansoni* tandem-repeat sequence was shown up to 58 weeks after treatment (397). In a cluster of travelers ($n = 13$) from Rwanda, all presenting with acute schistosomiasis, the same PCR approach using 2 ml of serum detected *Schistosoma* DNA in all 13 serum samples, whereas eggs of *S. mansoni* were found in 9 stool samples, and antischistosome antibodies were detected in 10 patients (383). In a multicenter study, the *Schistosoma* tandem-repeat sequence was amplified in 35 of 38 patients (92%) with acute schistosomiasis, compared to sensitivities of 70% and 24% using serology and microscopy, respectively (384). However, for patients ($n = 140$) who presented at an outpatient clinic in Antwerp, Belgium, using a real-time PCR developed to target the 28S rDNA sequence, *Schistosoma*-specific DNA was detected in all microscopy-positive stool ($n = 67$) and urine ($n = 4$) samples and additionally in microscopy-negative stool ($n = 9$) and urine ($n = 1$) samples, whereas the *Schistosoma* 28S target was amplified in only 2 of 38 serum samples of patients with confirmed schistosomiasis (398).

CESTODES

The impressive length of tapeworms has made them among the most well-known and evocative parasites to the general public. Although the pathology of intestinal cestodes is usually minor, if metacestode stages occur in human tissues, pathology and morbidity can be severe. Several species are known to infect humans, with *Taenia solium* (pork tapeworm), which can cause neurocysticercosis when metacestode stages develop in the brain after infection with eggs; *Taenia saginata* (beef tapeworm); *Hymenolepis nana* (dwarf tapeworm); and *Diphyllobothrium latum* (broad or fish tapeworm) being the most common (399).

Frequently, the diagnosis has already been made when patients notice the presence of proglottids in their feces. Parasitological differentiation based on morphological criteria might be difficult due to degeneration or the juvenile stage of the proglottids found. Microscopic detection of eggs in feces from patients with *Taenia* infections is known to be insensitive, as eggs expelled from proglottids are not equally distributed in the feces. Immunodiagnos-

TABLE 9 PCR-based assays for detection and molecular characterization of *Schistosoma* infections[f]

Species	Amplification method	Detection method	Target(s)	Sample type(s)	Reference
S. mansoni	PCR	Gel electrophoresis	*S. mansoni* tandem repeat	Adult worm	385
S. haematobium	PCR	Gel electrophoresis	*S. haematobium* tandem repeat	Adult worm	386
S. mansoni	PCR	Gel electrophoresis	*S. mansoni* tandem repeat	Stool, serum	387
S. mansoni	PCR[a]	Gel electrophoresis	*S. mansoni* tandem repeat	Stool	388
S. mansoni	PCR	Gel electrophoresis	Mitochondrial DNA	Stool	511
S. japonicum	PCR	Gel electrophoresis	Mitochondrial DNA	Stool	511
Schistosoma genus	PCR	Gel electrophoresis	28S	Urine	512
S. mansoni	PCR	Gel electrophoresis	28S	Urine	512
S. japonicum	PCR	Gel electrophoresis	28S	Urine	512
S. haematobium, *S. bovis*, *S. intercalatum*	PCR	Gel electrophoresis	ITS	Urine	512
S. mansoni	Real-time PCR	SYBR green	SSU rDNA		513
S. japonicum	Real-time PCR	SYBR green	*nad1*	Stool	514
S. mansoni	Multiplex real-time PCR	Hydrolysis probe	*cox1*	Stool	394
S. haematobium	Multiplex real-time PCR	Hydrolysis probe	*cox1*	Stool	394
Schistosoma genus	Real-time PCR	Hydrolysis probe	ITS2	Urine	458
S. japonicum	Real-time PCR	Hydrolysis probe	*nad1*	Stool	515
S. mansoni	PCR[a]	Gel electrophoresis	*S. mansoni* tandem repeat	Stool	516
Schistosoma genus	Real-time PCR	Hydrolysis probe	*S. mansoni* tandem repeat	Plasma	397
Schistosoma genus	Real-time PCR[c]	Hydrolysis probe	ITS2	Vaginal lavage	395
S. japonicum	LAMP	Visual	Retrotransposon SjR2 repeat	Serum	517
S. mansoni	PCR[a]	Gel electrophoresis	*S. mansoni* tandem repeat	Stool	518
Schistosoma genus	PCR-ELISA[a]	ELISA	*S. mansoni* tandem repeat	Stool	519
S. mansoni	PCR[a,b]	Gel electrophoresis	*S. mansoni* tandem repeat and 28S	Stool	520
S. haematobium	PCR[e]	Gel electrophoresis	*S. haematobium* tandem repeat	Cerebrospinal fluid	521
Schistosoma genus	Real-time PCR[d]	Hydrolysis probe	*S. mansoni* tandem repeat	Serum	383
S. haematobium	PCR[e]	Gel electrophoresis	*S. haematobium* tandem repeat	Urine	391
S. haematobium	PCR[e]	Gel electrophoresis	*S. haematobium* tandem repeat	Urine	392
S. japonicum	Nested PCR	Gel electrophoresis	*S. japonicum* repeat sequence	Serum	522
S. mansoni	PCR[a]	Gel electrophoresis	*S. mansoni* tandem repeat	Urine	523
S. japonicum	PCR	Gel electrophoresis	Retrotransposon SjR2 repeat	Stool	524
S. mansoni	PCR[a]	Gel electrophoresis	*S. mansoni* tandem repeat	Stool	525
S. mansoni	Nested PCR[a]	Gel electrophoresis	*S. mansoni* tandem repeat	Spiked stool	526
S. haematobium	PCR	Gel electrophoresis	*nad1* and *cox1*	Urine	393
Schistosoma genus	Real-time PCR	Hydrolysis probe	28S	Stool, urine, serum	398
Schistosoma genus	Real-time PCR[d]	Hydrolysis probe	*S. mansoni* tandem repeat	Plasma	384
Schistosoma genus	Real-time PCR[c]	Hydrolysis probe	ITS2	Urine	389
S. mansoni	PCR[a]	Gel electrophoresis	*S. mansoni* tandem repeat	Urine	527
Schistosoma genus	Real-time PCR[c]	Hydrolysis probe	ITS2	Urine, vaginal lavage	396

[a] PCR primers are described in reference 387.
[b] PCR primers are described in reference 512.
[c] PCR primers and probe are described in reference 458.
[d] PCR primers and probe are described in reference 397.
[e] PCR primers are described in reference 386.
[f] Abbreviations: RFLP, restriction fragment length polymorphism; SSU, small subunit; ITS, internal transcribed spacer; *cox1*, mitochondrial cytochrome *c* oxidase subunit I gene; *nad1*, NADH dehydrogenase subunit 1.

tic tests for the detection of *Taenia* antigens in stool samples have been used mainly in epidemiological studies, and DNA-based methods are used mainly for species discrimination (Table 10) (400, 401). Primarily, conventional (multiplex) PCR and PCR-RFLP designed based on the HDP1 repeat, HDP2, mitochondrial 12S rDNA, *cox1*, the pTsol9 repeat, and the Tso31 sequence have been used for species discrimination using DNA isolated from proglottids and have been tested only on small numbers of usually known positive and negative stool samples (Table 10). Recently, Bayesian modeling was used to estimate and compare the performances of microscopy, coproantigen ELISA, and multiplex real-time PCR for the detection of *Taenia* carriers by using stool samples ($n = 871$) collected in two Zambian communities where the disease is endemic. Specificities were 99.9%, 92.0%, and 99.0% and sensitivities were 82.5%, 84.5%, and 82.7% for microscopy, ELISA, and PCR, respectively (402). In a study comparing LAMP and a conventional multiplex PCR based on the *cox1* sequence using 43 known positive fecal samples, LAMP was more sensitive than PCR, 88.4% and 37.2%, respectively (403).

Molecular diagnostics have proven to be extremely valuable in the diagnosis of neurocysticercosis (404–406). In a group of 121 patients with confirmed neurocysticercosis, PCR targeting the pTsol9 repetitive element performed on DNA isolated from CSF samples showed the highest sensitivity (95.9%) compared to antibody detection by ELISA or immunoblotting and HP10 antigen detection by ELISA, with sensitivities of 90.1%, 53.7%, and 81%, respectively (405).

Species-specific PCRs for non-*Taenia* tapeworm species

TABLE 10 PCR-based assays for detection and molecular characterization of cestode infections[g]

Genus or species	Amplification method	Detection method	Target(s)	Sample type(s) (no. of samples)	Reference(s)
Diphyllobothrium genus	Multiplex PCR	Gel electrophoresis	*cox1*	Proglottids, eggs	407
T. saginata	PCR	Gel electrophoresis	HDP1	Proglottids	528, 529
T. solium, *T. saginata*	Multiplex PCR	Gel electrophoresis	HDP2	Proglottids	
T. solium, *T. saginata*	Multiplex PCR-RFLP[e]	Gel electrophoresis	HDP2	Proglottids	530
T. solium, *T. saginata*, *T. asiatica*	PCR-RFLP	Gel electrophoresis	Mitochondrial 12S rDNA	Proglottids	531
T. solium, *T. saginata*	Multiplex PCR[e]	Gel electrophoresis	HDP2	Stool	532
T. solium, *T. saginata*, *T. asiatica*	Multiplex PCR	Gel electrophoresis	*cox1*	Proglottids, stool (6)	533
T. saginata, *T. asiatica*	Multiplex PCR-RFLP	Gel electrophoresis	HDP2	Proglottids	534
T. solium, *T. saginata*, *T. asiatica*	Multiplex PCR[a]	Gel electrophoresis	*cox1*	Proglottids, stool (25)	535
T. solium, *T. saginata*	PCR-RFLP	Gel electrophoresis	*cox1*	Stool (12)	536
T. solium	PCR	Gel electrophoresis	Tsol9 repeat sequence	Cerebrospinal fluid	404
T. solium, *T. saginata*, *T. asiatica*	PCR-RFLP[b]	Gel electrophoresis	Mitochondrial 12S rDNA	Proglottids	537, 538
T. solium, *T. saginata*, *T. asiatica*	Multiplex PCR[a]	Gel electrophoresis	*cox1*	Proglottids, stool (19)	539
T. solium, *T. saginata*, *T. asiatica*	Multiplex PCR[a]	Gel electrophoresis	*cox1*	Proglottids	540
T. solium, *T. saginata*, *T. asiatica*	Multiplex PCR[a]	Gel electrophoresis	*cox1*	Proglottids	541
T. solium	Nested PCR	Gel electrophoresis	Tsol31	Stool (155)	542
T. solium	Nested PCR	Gel electrophoresis	HDP2	Cerebrospinal fluid	543
Taenia genus	PCR	Sequencing	ITS1	Cerebrospinal fluid	425
T. solium, *T. saginata*, *T. asiatica*	Multiplex PCR	Gel electrophoresis	*cox1*	Proglottids	544
T. solium, *T. saginata*, *T. asiatica*	LAMP	Gel electrophoresis	Clp, *cox1*	Proglottids, cysticerci, stool (6)	545
T. solium, *T. saginata*, *T. asiatica*	LAMP[f]	Gel electrophoresis	Clp, *cox1*	Stool (43)	403
T. solium, *T. saginata*, *T. asiatica*	PCR	Gel electrophoresis	HDP2	Proglottids	546
T. solium, *T. saginata*, *T. asiatica*	Multiplex PCR[a]	Gel electrophoresis	*cox1*	Proglottids	547
T. solium	PCR[d]	Gel electrophoresis	Tsol9 repeat sequence	Cerebrospinal fluid	405
T. solium	Real-time PCR	LNA hydrolysis probe	Tsol9 repeat sequence	Cerebrospinal fluid	406
T. solium, *T. saginata*, *T. asiatica*	Multiplex PCR[c]	Gel electrophoresis	*cox1*	Proglottids, eggs	548
T. solium, *T. saginata*, *T. asiatica*	PCR-RFLP[b]	Gel electrophoresis	Mitochondrial 12S rDNA	Proglottids	537
T. solium, *T. saginata*, *T. asiatica*	PCR-RFLP[b]	Gel electrophoresis	Mitochondrial 12S rDNA	Proglottids	549
T. solium, *T. saginata*, *T. asiatica*	Multiplex PCR[c]	Gel electrophoresis	*cox1*	Proglottids	550
T. solium, *T. saginata*	Real-time multiplex PCR	Hydrolysis probe	ITS1	Stool (817)	402

[a] PCR primers are described in reference 533.
[b] PCR primers are described in reference 531.
[c] PCR primers are described in reference 544.
[d] PCR primers are described in reference 404.
[e] PCR primers are described in references 528 and 529.
[f] LAMP primers were described previously (545).
[g] Abbreviations: RFLP, restriction fragment length polymorphism; LNA, locked nucleic acid; SSU, small subunit; ITS, internal transcribed spacer; *cox1*, mitochondrial cytochrome *c* oxidase subunit I gene; Clp, cathepsin L-like cysteine peptidase; HDP1, *T. saginata*-specific repetitive sequence; HDP2, cestode-specific sequence.

have not been widely used. A multiplex PCR targeting the *cox1* sequence with a general reverse primer and four species-specific forward primers was used for the specific amplification of *Diphyllobothrium latum*, *D. dendriticum*, *D. pacificum*, and *D. nihonkaiense* DNAs from adult worms, plerocercoid larvae, and eggs (407).

PCR followed by sequencing using universal primers to amplify the ribosomal ITS1, ITS2, 5.8S, and SSU rDNA sequences or mitochondrial genes is used to identify and confirm the species identity of tapeworms found in individual cases as well as interspecies variation to construct phylogenetic relationships (408–411). Sequence analysis of the ITS1, *cox1*, and paramyosin gene sequences from *H. nana* isolates from northwest Australia provided genetic support that the life cycle involves mainly human-to-human transmission (412).

CONCLUDING REMARKS

A large number of studies using a variety of nucleic acid-based techniques have contributed to our understanding of the genetic diversity, epidemiology, and clinical relevance of intestinal parasites. For obvious reasons, in a routine setting, standardization and harmonization of protocols are essential for cost-effective implementation of these new techniques. A syndrome-driven approach that includes the detection of the most likely pathogens in a particular setting using a single overall technique can be facilitated by the use of multiplex real-time PCR. Such an approach offers a highly sensitive and specific diagnostic alternative to labor-intensive microscopy in clinical laboratory practice for the diagnosis of intestinal pathogens. As molecular diagnostic facilities in general microbiology laboratories are already widely available, an increasing number of laboratories have implemented real-time PCR for the first-line diagnosis of intestinal parasitic infections. The implementation of nucleic acid-based tests is particularly useful for diarrhea-causing protists when used in combination with additional panels for the detection of bacterial and viral enteritis agents (106, 413). In comparison with microscopic examination, the rate of detection of parasitic infections when the targets are included in a multiplex PCR is considerably high (27,

87, 104, 107, 162, 414). However, parasites that are not included as targets in the multiplex PCR will not be detected, which is one of the arguments that is frequently raised against the use of molecular diagnostics in parasitology. Although true, it is good to realize that, diarrhea-causing protists aside, the prevalence of other parasitic pathogens detected by microscopy of stool samples submitted to most general microbiology laboratories in industrialized countries is extremely low (27, 162, 328, 413, 415–418). Even in travelers, additional parasites are found by microscopy in only a small minority of cases, with the highest detection rates in travelers to high-risk areas (107, 419). The use of additional diagnostic methods for the detection of those parasitic infections that are not included in a standard fecal PCR panel may therefore be limited to a select group of patients. For some laboratories, this would probably mean that there are only a few cases in which additional tests are needed, whereas, for example, in laboratories located in large metropolitan areas with large immigrant populations and serving many travelers, it would be more efficient to employ a more elaborate PCR panel and/or to perform additional microscopy on all samples submitted. This approach is similar to algorithms that are used where PCR is not a method of choice for the routine diagnosis of parasitic infections. Decisions on a standard screening technique to employ are based on the general patient population profile of the laboratory, including travel (immigration and adoption), immune status, eosinophilia, larva currens, persistent symptoms, and so on, with or without additional techniques for the diagnosis of other infections (27, 417, 420). For example, in immunocompromised patients with diarrhea, an additional method for the detection of opportunistic pathogens such as microsporidia and *C. belli* may be appropriate. This could be performed by optical white staining and acid-fast staining, respectively, or by using a multiplex PCR for the detection of both pathogens.

Molecular techniques are ideally suited for automation, and following the rapid growth of nucleic acid-based techniques for a large variety of targets, automation of the diagnostic process is currently being implemented in many laboratories. Automation of nucleic acid isolation, PCR setup, and PCR, when integrated through middleware with a laboratory information management system (LIMS), facilitates a rapid sample-to-answer process. This could be seen by some as another step in the separation of the laboratory, the clinician, and the individual patient. However, in this process, primary screening through a general algorithm can be followed by additional techniques decided upon during medical authorization based on individual clinical records and patient history.

Obviously, just as quality assessment schemes are common practice for microscopy-based diagnosis, the application of molecular diagnostics requires quality assurance as well. Initiatives to this end have been taken (421). Since 2012, Quality Control for Molecular Diagnostics (QCMD) (http://www.qcmd.org/) provides a yearly quality assessment scheme for gastrointestinal diseases, including a panel for intestinal protozoa. The Dutch Foundation for Quality Assessment in Medical Laboratories (SKML) (http://www.skml.nl/) has started an international scheme for molecular diagnosis of intestinal protozoa as well.

FUTURE DIRECTIONS

Throughout the world, including developing countries, real-time PCR is available in an increasing number of research centers, and molecular diagnosis of parasitic infections can be applied to large-scale epidemiological and more fundamental research. One major advantage is the possibility of an integrated high-throughput approach for the detection of a range of parasitic, bacterial, and viral targets using the same technique and for which samples can be collected and transported to central laboratories. Moreover, samples can be stored and used later for testing of additional targets when new research questions arise or, for example, for detection of mutations that are associated with drug resistance. Although, at first glance, PCR is more expensive than conventional microscopy-based techniques, this must be weighed against the complexities of organizing the prolonged stay of a large team of technicians and support staff in the field, sometimes under primitive conditions.

In a number of European countries, (automated) multiplex PCRs, which are usually in-house tests, are routinely used in clinical diagnostic laboratories. Commercial multiplex PCRs for the detection of a range of enteropathogens, which are already optimized and validated for a variety of instruments, as well as kits to be used on more extended multiplex platforms, such as Luminex, and variations on microarrays are all available (413, 422–424). This trend will make it easier for laboratories that are just starting to use molecular diagnostics to catch up with the rapid developments in this field. The first FDA-approved multiplex gastroenteric panel will lead to the more widespread use of nucleic acid-based tests in the United States as well (Table 2). Test formats that combine sample processing and DNA amplification of enteropathogens for single or multiple samples into one device have recently been launched or are still under development (e.g., BDmax [Becton, Dickinson and Company] and FilmArray [Biofire Diagnostics]) and will make it feasible for smaller laboratories to implement nucleic acid-based diagnostics. The cost-effectiveness of such an approach compared to in-house or commercially available high-throughput platforms, however, needs to be investigated. Another interesting approach is the use of an extended-range PCR screening strategy for the detection of bacterial, viral, fungal, and parasitic organisms in complex cases with a broad differential diagnosis; this has been reported in a case of cystoisosporiasis and in a case of neurocysticercosis (211, 425).

While there is no doubt that molecular diagnostic methods will continue to rely on the detection of genus- or species-specific DNA or RNA, new-generation sequencing platforms, which as yet are used primarily for research, will become more user-friendly and will be applied in a diagnostic setting. This will enable, for example, screening of fecal DNAs using broad-specificity primers for in-depth analysis of the microbially diverse populations present in such samples (426). In addition to targeting microorganisms, the host factors potentially involved in pathogenesis can also be explored.

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