

Review

New Technologies for Detection of Enteric Parasites

Una Ryan,^{1,*} Andrea Papparini,¹ and Charlotte Oskam¹

Enteric parasites are major contributors to the global diarrhoeal disease load, infecting >67.2 million people. Their prevalence and clinical impact, however, are underestimated due to lack of adequate detection, which is largely still based on microscopy, particularly in developing countries. New commercially available enteric panel assays, which detect parasites (as well as bacteria and/or viruses) using multiplex PCR, offer enhanced sensitivity and specificity as well as the ability to detect mixed infections, and will play an important role in epidemiological surveillance and outbreak investigations. A major limitation of these technologies, however, particularly for developing countries, is the costs involved. Emerging technologies for low-resource, point-of-care (POC) settings have the potential to dramatically improve the cost and accuracy of enteric parasite detection in the future.

Global Impact of Enteric Parasites

Globally, there are nearly 1.7 billion reported cases of diarrhoeal disease every year and ~760 000 deaths in children under 5 years (e.g., [1], <http://www.who.int/mediacentre/factsheets/fs330/en/>). The incidence is highest in young children, due to immunological immaturity, transition from breastfeeding to potentially contaminated foods, and/or poor sanitation and water treatment [1]. In addition to mortality, diarrhoeal diseases cause significant morbidity and have a lasting negative impact on the growth, development, and cognition of children [1]. The socioeconomic burden on health services has been estimated at 72.8 million disability-adjusted life years (DALYs) worldwide (e.g., http://www.who.int/healthinfo/global_burden_disease/GBD_report_2004update_full.pdf). In different countries, the true costs are unknown but the approximate annual costs have been estimated at €600 million in The Netherlands [2], AUD\$1.2 billion in Australia [3], Can\$ 3.7 billion in Canada [4] and £1.5 billion in the UK (e.g., http://www.food.gov.uk/sites/default/files/multimedia/pdfs/publication/cstar_2013.pdf).

Causes of diarrhoea include viruses, bacteria, protozoa, and occasionally worms; however, the cause of infectious diarrhoea is not determined in up to 80% of cases, resulting in inappropriate use of antibiotics [5,6]. Therefore, more effective diagnostic tools are urgently required. Enteric protozoan parasites are known to be among the major contributors to the global diarrhoeal disease load [7,8] and are the focus of this review. For example, a World Health Organization study recently reported that enteric protozoa contributed to 67.2 million illnesses or 492 000 DALYs [9,10]. Although the majority of infections and deaths from enteric parasites affect people in developing countries, the emergence of immunocompromised populations, combined with an increase in life expectancy, has resulted in enteric parasites causing significant illness in developed countries and may cause a greater economic burden due to higher income, medical, and treatment costs [11]. This review discusses current commercially available and emerging diagnostic assays for protozoan parasites and their use in developing and developed countries.

Trends

Accurate diagnosis of diarrhoea caused by enteric parasites requires rapid, cost-effective, scalable, high-throughput, reproducible, and sensitive detection methods.

Mixed infections are common, requiring clinicians and the detection methods they employ to screen for multiple pathogens.

Current detection methods are labour-intensive, time-consuming, lack sensitivity and specificity, and fail to detect mixed infections.

Gastrointestinal panel (GiP) assays based on multiplex PCR afford the opportunity to detect mixed infections and adhere to the requirements for diarrhoeal disease diagnosis.

Costs associated with GiP assays are still unaffordable for laboratories, particularly in developing countries.

More cost-effective, field-deployable technologies are required.

¹School of Veterinary and Life Sciences, Murdoch University, WA, 6150, Australia

*Correspondence: Una.Ryan@murdoch.edu.au (U. Ryan).

Protozoan Causes of Diarrhoea

The prevalence and clinical impact of enteric parasites are underestimated due to lack of adequate detection and surveillance systems in developing countries, particularly as enteric protozoa are often ignored by surveillance systems. *Entamoeba histolytica*, *Cryptosporidium* spp., *Giardia duodenalis*, and *Cyclospora cayetanensis*, however, are recognised as important causes of diarrhoea in developing regions such as Asia and sub-Saharan Africa [7,12–16]. In developed countries, in addition to these protozoan parasites, *Dientamoeba fragilis* and *Blastocystis* sp. are frequently isolated [7,17]. These enteric protozoan parasites cause a range of clinical symptoms in addition to diarrhoea (Box 1). The pathogenic potential of *Blastocystis* remains controversial, however, as although >1 billion individuals worldwide are colonised by *Blastocystis*, asymptomatic colonisation is common and pathogenicity has yet to be robustly demonstrated *in vivo* [17].

Limitations of Current Detection Methods

Surveillance systems such as the US Centre for Disease Control (CDC), European Centre for Disease Control (ECDC), and the Australian National Notifiable Diseases Surveillance System (NNDSS) are in place in developed countries. However, all surveillance systems underestimate the prevalence of enteric parasites as (i) it is estimated that less than 10% of individuals with gastroenteritis visit their local doctor, and of these, less than 10% have a faecal specimen collected [18–20], (ii) not all individuals presenting with gastroenteritis will have faecal samples tested for microorganisms [18,19], and (iii) there is a lack of available sensitive diagnostic techniques to detect enteric parasites in clinical specimens, which results in subclinical

Box 1. Clinical Symptoms Associated with Enteric Protozoan Parasites

Cryptosporidium hominis and *Cryptosporidium parvum* are responsible for the majority of cryptosporidiosis infections in humans and are a major cause of moderate to severe diarrhoea worldwide, second only to rotavirus, with >2.9 million cases annually in children aged <24 months in sub-Saharan Africa [8,12,14,15,113,114]. Symptoms include abdominal pain, fever, vomiting, malabsorption, and usually self-limiting diarrhoea. In children, it is also associated with malnutrition, growth retardation, impaired immune response, and cognitive deficits [14,15,114].

Giardia duodenalis causes acute, watery, usually self-limiting diarrhoea (giardiasis) in >280 million people annually and, along with *Cryptosporidium*, is a common cause of waterborne outbreaks [13,115]. The Global Enteric Multicenter Study (GEMS) study reported that *Giardia* was not significantly positively associated with moderate-to-severe diarrhoea [12]. However, a meta-analysis of endemic paediatric giardiasis concluded that although giardiasis is associated with protection from acute diarrhoea, it is an increased risk for persistent diarrhoea [13]. Chronic infections can result in weight loss and malabsorption [116], and can elicit protracted postinfectious syndromes, including irritable bowel syndrome and chronic fatigue [102]. Infections are also associated with stunting (low height for age), wasting (low weight for height), and cognitive impairment in children in developing countries [16,116].

Entamoeba histolytica is a protozoan parasite and the causative agent of amoebiasis; it results in ~100 000 human deaths annually [117]. The GEMS study identified *E. histolytica* as one of the top 10 causative agents of moderate to severe diarrhoea in two of their seven study sites across Africa and Southeast Asia [12]. *E. histolytica* is the only *Entamoeba* species thought to be pathogenic, but infection with other morphologically indistinguishable *Entamoeba* species, such as *Entamoeba dispar* and *Entamoeba moshkovskii*, complicates diagnosis. However, a recent report suggests that *E. dispar* is capable of causing lesions [118]. Transmission occurs mainly via ingestion of food and water contaminated with amoebic cysts. Clinical symptoms of *E. histolytica* infection range widely from asymptomatic to severe symptoms, including dysentery and extraintestinal abscesses [117].

Symptoms of cyclosporiasis, caused by *Cyclospora cayetanensis*, include diarrhoea, fatigue, and abdominal cramps, which are most likely reported between 1 and 2 weeks after infection [16]. Other symptoms are general malaise, lack of energy, loss of appetite, mild fever, nausea, flatulence, and abdominal cramps [16]. It is endemic in developing countries and associated with sporadic outbreaks in developing countries [16,119].

Dientamoeba fragilis is one of the smaller parasites that can live in the human gut and, unlike most other enteric protozoa, its life cycle has no cyst stage; thus, infection between humans occurs during the trophozoite stage. The infection (dientamoebiasis) causes diarrhoea, chronic abdominal pain, chronic fatigue, and it equals or exceeds the incidence of giardiasis [120,121].

infections not being diagnosed [18]. These problems are compounded in developing countries due to the remoteness of communities, lack of transport and communication infrastructures, and a shortage of skilled health care workers and laboratory facilities to ensure accurate and rapid diagnosis (e.g., http://apps.who.int/iris/bitstream/10665/170250/1/9789240694439_eng.pdf).

Microscopic ova and parasite examination (O & P) (i.e., wet mount after stool concentration and different staining methods) is still the traditional method for stool parasite testing in developing countries and many developed countries [18,21–23]. In resource-poor health systems, the advantages of microscopy are that minimal equipment and reagents are required. However, O & P is labour-intensive, time-consuming, lacks sensitivity and specificity, and requires a high level of skill for optimal interpretation. There is also a lack of skilled technologists capable of reliably evaluating O & P in both developing and developed countries ([18], e.g., http://apps.who.int/iris/bitstream/10665/170250/1/9789240694439_eng.pdf). Sensitivities as low as 54.8% and 66.4% by microscopy have been reported for *Cryptosporidium* and *Giardia* respectively [18]. Other factors that influence the sensitivity of parasite examinations include sporadic shedding (requiring the examination of multiple stool specimens), patient medications, specimen collection interval, and the preservation of stool prior to testing [21]. Microscopy

Table 1. Sensitivities and Specificities of Currently Used Immunological Diagnostic Tests for Enteric Parasites

Parasite	Test name	Supplier	Specificity	Sensitivity	Refs
<i>Cryptosporidium</i>	Crypto-Strip	Coris BioConcept, Belgium	100%	47.2%	[122]
	RIDA [®] QUICK	R-biopharm Diagnostic, Germany	98%	62.4%	[122]
	RIDA [®] QUICK Combi	R-biopharm Diagnostic, Germany	100%	100%	[123]
	Remel-Xpect	Remel Inc., USA	100%	68.8%	[122]
	ImmunocardSTAT [®] C/ G	Meridian Bioscience Inc., USA	96.6–100%	5.5–70.6%	[122,124]
	ImmunoCard STAT! [®] CGE	Meridian Bioscience Inc., USA	45.6–100%	100%	[23,24,123]
	<i>Cryptosporidium</i> and <i>Giardia</i> Duo-Strip	Coris BioConcept, Belgium	100%	91.7%	[123]
<i>Giardia</i>	ImmunocardSTAT [®] C/ G	Meridian Bioscience Inc., USA	96.6%	63.6%	[124]
	ImmunoCard STAT! [®] CGE	Meridian Bioscience Inc., USA	100%	83%	[123]
	<i>Cryptosporidium</i> and <i>Giardia</i> Duo-Strip	Coris BioConcept, Belgium	100%	58%	[123]
	RIDA [®] QUICK Combi	R-biopharm Diagnostic, Germany	100%	83%	[123]
<i>Entamoeba</i>	Quik Chek	Techlab, USA	100%	98%	[125]
	<i>E. histolytica</i> II ELISA	Techlab, USA	99%	0%	[126]
	<i>Entamoeba</i> CELISA PATH kit	Cellabs, Australia	100%	28%	[126]
	RIDA [®] QUICK Combi	R-biopharm Diagnostic, Germany	100%	88%	[123]
	ImmunoCard STAT! [®] CGE	Meridian Bioscience Inc., USA	80%	100%	[123]

detection using fluorescent antibody staining has increased the sensitivity and specificity of detection of enteric protozoa [23], but is still time consuming and requires a skilled microscopist. To overcome these limitations, rapid faecal antigen detection tests for *Giardia*, *Cryptosporidium* spp., and *E. histolytica* have been developed and approved by the US Food and Drug Administration (FDA) and are widely used in the USA. These include immunochromatographic and enzyme-linked immunosorbent assay (ELISA) (Table 1). Despite this, in practice the specificities and particularly the sensitivities of these tests are highly variable, resulting in false positives and false negatives (Table 1). For example, for *Cryptosporidium*, some immunochromatographic assays have such low sensitivity and specificity, that the US Council of State and Territorial Epidemiologists (CSTE) now exclude cases diagnosed as positive for *Cryptosporidium* by immunochromatographic assays, from cryptosporidiosis surveillance data, and specifies that cases diagnosed with these laboratory tests be considered probable rather than confirmed [23,24], (e.g., <http://c.ymcdn.com/sites/www.cste.org/resource/resmgr/PS/11-ID-14.pdf>). False positives in immunodetection assays can arise from not reading the result at the prescribed time, inconsistency between test operators and readers, incorrect sample preservation, improper dilution of sample, use of out-of-date tests, and improper storage temperature of tests and/or specimens [23]. No antigen-based diagnostics have been commercially developed to date, to aid with laboratory identification of *Cyclospora* and *Dientamoeba* [18].

Molecular Testing

As a result of the limitations of microscopic and immunological assays, DNA-based detection methods have been developed for enteric parasites. This approach exhibits numerous advantages over traditional methods, such as an increased sensitivity and specificity, ability to combine multiple targets in one multiplex assay, the possibility for quantitation and molecular typing, and a rapid turnaround time, particularly when PCR is coupled to automated DNA extraction [25,26]. Despite this, until recently, molecular detection has not been widely adopted in commercial diagnostic laboratories. However, in developed countries, increasing labour costs for microscopists, including the need for rapid results, continuous training and the low sensitivity and specificity of microscopy and antigen testing has meant that there is a growing willingness of well-equipped laboratories to adopt DNA-based technologies for routine diagnostic procedures, replacing microscopy [26]. In developing countries, with the increased focus and funding on the burden of enteric pathogens such as the Global Enteric Multicenter Study (GEMS), there is an even greater need for more sensitive and high-throughput diagnostic assays, particularly as improved diagnostics can help prevent transmission and provide active surveillance. Molecular assays are, however, particularly sensitive to the quality and purity of the starting DNA material, so the choice of an efficient DNA extraction method is a critical step, as performance differences have been observed between different commercially available DNA-extraction kits, depending on the parasite and the infection burden [27]. The different types of molecular test available for intestinal parasites have been recently reviewed [28–32].

Recent Diagnostic Molecular Tests

The development of quantitative PCR (qPCR)-based detection methods has improved the detection of protozoan parasites as they are amenable to automation and have a higher throughput capacity [25]. Most new tests are based on this technique, and several FDA-cleared molecular assays, based on multiplex PCR assays, have recently become available for simultaneous detection and identification of common enteric protozoan parasites (as well as bacteria and/or viruses) (Table 2; Figure 1, Key Figure). These include the BD MAXTM Enteric Parasite Panel (EPP) (Becton, Dickinson and Company, USA), the Luminex xTAG[®] Gastrointestinal Pathogen Panel (Luminex Corporation, Toronto, Canada), the NanoCHIP[®] GIP (Savyon[®] Diagnostics Ltd, Israel), the Biofire FilmArrayTM Gastrointestinal Panel (BioFire

Table 2. New Commercially Available DNA-Based Technologies for the Detection of Enteric Parasites, Based on Multiplex PCR

Method	Parasites detected	Specificity	Sensitivity	Advantages	Disadvantages	Refs
BD Max parasitic panel (EPP)	<i>Giardia duodenalis</i> , <i>Cryptosporidium hominis</i> , <i>Cryptosporidium parvum</i> , and <i>Entamoeba histolytica</i>	99.5% (<i>G. duodenalis</i>), 99.6% (<i>C. parvum</i> or <i>C. hominis</i>), and 100% and (<i>E. histolytica</i>)	98.2% (<i>G. duodenalis</i>), 95.5% (<i>C. parvum</i> or <i>C. hominis</i>), and 100% (<i>E. histolytica</i>)	No cross reaction with nonpathogenic <i>Entamoeba</i> sp. Can be performed with specimens submitted in either 10% formalin or unpreserved.	Effects of prolonged transport or storage were not evaluated	[33,34]
Luminex xTAG [®] Gastrointestinal Pathogen Panel (GPP)	12 bacterial and viral pathogens and 3 parasites: <i>G. duodenalis</i> , <i>Cryptosporidium</i> , <i>E. histolytica</i>	99–100% for <i>G. duodenalis</i> , 100% for <i>Cryptosporidium</i> , 89–100% for <i>E. histolytica</i> and variable specificity for other pathogens	95–100% for <i>G. duodenalis</i> , 95–100% for <i>Cryptosporidium</i> , 100% for <i>E. histolytica</i> and variable sensitivity for other pathogens	Increased detection of mixed infections. Shorter turn-around time. Can detect aetiological agents not requested by physicians. Can detect mixed infections.	Cost (US\$75 per sample) Open system – potential for contamination.	[36,38,35,39–56]
NanoCHIP [®] Gastrointestinal Panel (GIP)	<i>G. duodenalis</i> , <i>Cryptosporidium</i> spp., <i>E. histolytica</i> , <i>E. dispar</i> , <i>Dientamoeba fragilis</i> , <i>Blastocystis hominis</i> and 3 bacterial species	Up to 100% – further testing needed.	Detection limit of 5×10^3 for parasites. 100% for <i>Giardia</i> sp., <i>Cryptosporidium</i> spp., <i>E. histolytica</i> , <i>E. dispar</i> ; 98% for <i>D. fragilis</i> ; 95% for <i>Blastocystis</i> spp. High sensitivity for other pathogens.	Less than US\$30 per sample. Increased efficiency in detecting mixed infections over conventional methods. Higher sensitivity and detection yield. Reduced hands-on time and workload. High throughput.	Novel or emerging enteric parasites may go undiagnosed.	[57]
Biofire FilmArray [™] Gastrointestinal Panel	14 bacterial, 5 viral and 4 parasites: <i>G. duodenalis</i> , <i>Cryptosporidium</i> , <i>E. histolytica</i> , and <i>Cyclospora cayetanensis</i> .	99.5–100% for <i>G. duodenalis</i> , 99.6–100% for <i>Cryptosporidium</i> , 89–100% for <i>E. histolytica</i> , 100% for <i>C. cayetanensis</i> and variable specificity for other pathogens	100% for <i>G. duodenalis</i> , 100% for <i>Cryptosporidium</i> , 100% for <i>E. histolytica</i> , 100% for <i>Cyclospora cayetanensis</i> and variable sensitivity for other pathogens	Detection of pathogens not routinely ordered, e. g., <i>Cyclospora</i> . Able to detect emerging <i>Cryptosporidium</i> pathogens in humans including <i>C. ubiquitum</i> and <i>C. felis</i> . Can detect unculturable <i>Shigella</i> .	23.8–80% sensitivity for <i>Aeromonas</i> .	[43,58–60,62]
EasyScreen [™]	<i>G. duodenalis</i> , <i>Cryptosporidium</i> spp., <i>Entamoeba</i> complex, <i>D. fragilis</i> and <i>Blastocystis</i> spp.,	100%	92% for <i>G. duodenalis</i> , 100% for <i>Cryptosporidium</i> , 92% for <i>Entamoeba</i> complex, 92% for <i>D. fragilis</i> , and 96% for <i>Blastocystis</i> .	Contains an extraction and separate amplification control for the detection of PCR inhibition	Detects nonpathogenic as well as pathogenic <i>Entamoeba</i> spp.	[61]

Diagnostics, Salt Lake City, UT), the Verigene Enteric Pathogens (EP) test (Luminex) and the EasyScreen[™] Enteric Parasite Detection Kit (Genetic Signatures, Sydney, Australia).

The BD MAX[™] Enteric Parasite Panel (EPP) is a multiplex PCR assay that detects *G. duodenalis*, *Cryptosporidium hominis*, *Cryptosporidium parvum*, and *E. histolytica* in formalin-fixed and unpreserved stool specimens. The BD MAX[™] instrument performs automated extraction and amplification, with the amplified DNA detected using hydrolysis (TaqMan[®]) probes, with a turnaround time of ~3.5 h. A multicentre evaluation involving four US laboratories that

Key Figure

Current and Emerging Detection Methods for Human Enteric Protozoan Parasites

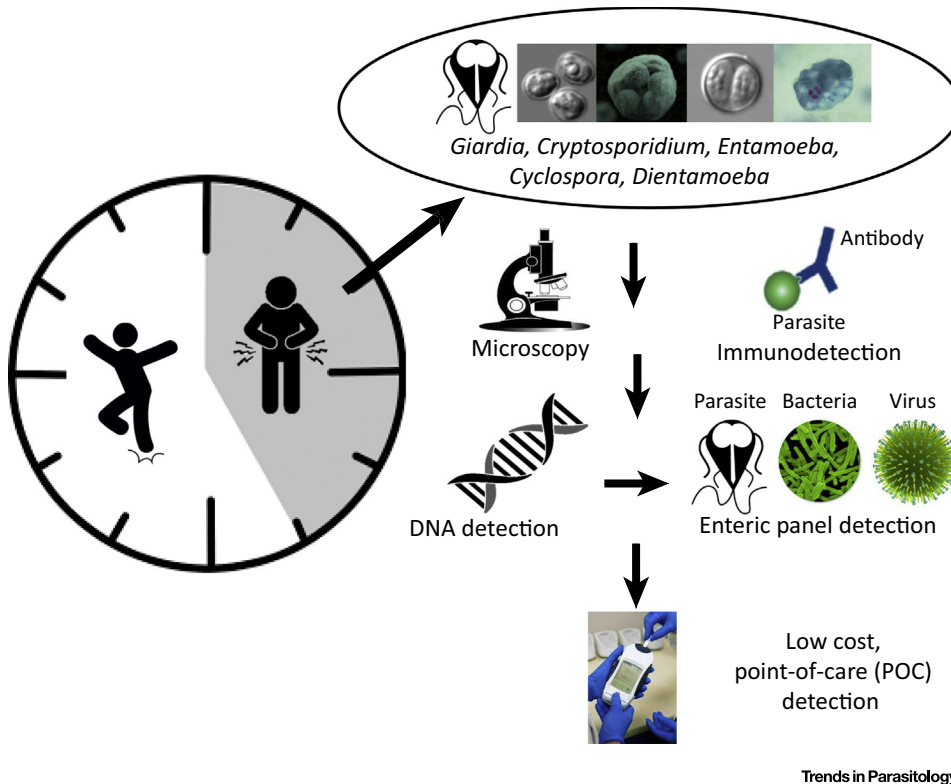


Figure 1. As rapid and sensitive diagnosis is crucial for effective management of enteric infections, detection methods are moving from traditional microscopy and immune-detection to high-throughput DNA-based enteric panel detection assays that detect a wide range of enteric pathogens, including parasites, bacteria, and viruses. The cost of many of these detection methods, however, prohibit their use in developing countries. The ultimate goal is point-of-care (POC) testing that is Affordable, Sensitive, Specific, User-friendly, Rapid and Robust, Equipment-free, and Deliverable to end-users (ASSURED).

perform clinical testing, was conducted on 2495 samples and the results were compared to those from PCR and bidirectional sequencing, as well as direct fluorescent antibody (*G. duodenalis* and *C. parvum/C. hominis*) or trichrome stain (*E. histolytica*) [33]. Overall, the sensitivity and specificity was 98.2% and 99.5% for *G. duodenalis*, 95.5% and 99.6% for *C. parvum* or *C. hominis*, and 100% and 100% for *E. histolytica*, respectively [33]. An earlier analysis on a small number of clinical samples reported a sensitivity of 66.7% for *G. duodenalis* [34] but the fluorescent cutoff has subsequently been optimised by the company to improve sensitivity [34]. The cost of the BD MAX™ kit is, however, expensive for routine diagnostic use at AUD\$50/sample.

The Luminex xTAG® gastrointestinal pathogen panel (GIP) is a qualitative bead-based multiplex PCR assay able to simultaneously detect, in a single human stool sample, 15 different pathogens: *Cryptosporidium*, *E. histolytica*, *G. duodenalis*, *Campylobacter*, *Clostridium difficile* (Toxins A/B), Enterotoxigenic *E. coli* (ETEC), *Escherichia coli* O157, *Salmonella*, *Shigella*,

Shiga-like toxin-producing *E. coli* (STEC), *Vibrio cholera* (cholera toxin gene), *Yersinia enterocolitica*, adenovirus 40/41, norovirus GI/GII, and rotavirus A. The Luminex xTAG[®] system can process up to 24 stool samples in a single batch and involves a multiplex PCR with the PCR products hybridised to uniquely tagged microspheres with a turnaround time of approximately 5 h. It has been extensively reviewed in different settings and countries [35–56]. The sensitivity and specificity of the assay has been reported to be 95–100% and 99–100% for *G. duodenalis*, 95–100% and 100% for *Cryptosporidium*, and 100% and 89–100% for *E. histolytica*, respectively, and variable specificity and sensitivity for other pathogens [35–56]. One study reported that, for *Salmonella enterica*, the sensitivity dropped from 84% to 46% for fresh extracts due to extraction issues [36]. In addition, the Luminex platform is an open system (DNA extraction is not automated), and therefore the possibility exists for amplicon contamination. The Luminex xTAG[®] system is also very expensive (currently ~US\$75/sample, as of January 2017). However, an 8-month cost-benefit analysis for detection of infectious gastroenteritis in 800 hospitalised patients in the UK, in comparison with conventional laboratory testing (based on a combination of culture, microscopy, and enzyme immunoassay), reported that despite a 62% increase in laboratory costs, there were net savings as test-negative patients could be moved out from isolation rooms, thus bringing about a significant reduction in isolation room costs [47]. This has been queried by others who have commented that releasing patients from isolation just on the basis of this multiplex PCR result could be problematic as there is always a proportion of false-negatives, and false-negative results [50]. In addition, the use of the Luminex xTAG system on all stool specimens, not just on hospital specimens, would result in a large increase in costs [51].

The NanoCHIP[®] GIP (Savyon[®] Diagnostics Ltd, Israel) is an automated multiplex PCR assay performed on a molecular electronic microarray system, which detects bacteria (*Salmonella* spp., *Shigella* spp., and *Campylobacter* spp.), and the protozoan parasites *E. histolytica*, *E. dispar*, *Giardia* sp., *Cryptosporidium* spp., *Blastocystis* spp., and *D. fragilis* [57]. The system was designed for maximum high-throughput use in large diagnostic laboratories, with the capacity to automate the analysis of 96 samples simultaneously from DNA extraction through to parasite detection. This reduces the standard sample-to-result time from conventional methods (48–72 h) to next-day results with the NanoCHIP[®] GIP system. Based on retrospective and prospective sample analyses, the NanoCHIP GIP has comparable performances to the Luminex xTAG, but outperforms conventional methods, revealing higher sensitivity (10^3 to 10^5) and detection yield, and the detection of mixed infections that were previously undiagnosed [36,57]. When the NanoCHIP[®] GIP was compared to conventional and qPCR tests for both retrospective and prospective studies, there was a positive agreement of 100% for the detection of *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., and *Giardia* sp., and 98% and 95% for *D. fragilis* and *Blastocystis* spp., respectively. Moreover, this technique required less hands-on time due to the automation of the workflow, which reduces human-associated errors. The cost of the system per sample is around US\$30 per sample, proving more cost effective than the Luminex xTAG.

The multiplex Biofire FilmArray[™] Gastrointestinal Panel can detect 22 pathogens: seven bacteria (*Aeromonas* spp., *Campylobacter* spp. [*C. jejuni*, *C. coli*, and *C. upsaliensis*], *C. difficile* toxin A/B, *Plesiomonas shigelloides*, *Salmonella* spp., *Vibrio* spp. [*V. parahaemolyticus*, *V. vulnificus*, and *V. cholerae* with specific detection of *V. cholerae*], and *Y. enterocolitica*), six diarrheagenic *Shigella* spp./*E. coli* (enteroaggregative *E. coli* [EAEC], enteropathogenic *E. coli* [EPEC], enteroinvasive *E. coli* [EIEC]/*Shigella* spp., enterotoxigenic *E. coli* [ETEC], Shiga-like toxin-producing *E. coli* [STEC- *E. coli* O157]), four enteric parasites (*Cryptosporidium*, *E. histolytica*, *G. duodenalis*, *C. cayetanensis*), and five viruses (adenovirus F 40/41, astrovirus, norovirus GI/GII, rotavirus A, and sapovirus). The closed system can test one stool sample in approximately 1 h, with 2 min hands-on time, and conducts DNA extraction

and nested multiplex PCR followed by endpoint melting curve analysis with the FilmArray software. The sensitivity and specificity of the assay has been reported as 100% and 99.5–100% for *G. duodenalis*, 95–100%, and 99.6–100% for *Cryptosporidium*, 100% and 89–100 for *E. histolytica*, 100% and 100% for *C. cayetanensis*, respectively, and variable specificity for other pathogens [43,54,58,59]. The assay is very expensive (~US\$155/sample) [60]. One study highlighted the benefit of a panel-based approach to diagnostic testing as a national outbreak of cyclosporiasis was detected during a research trial of the FilmArray system [59]. The outbreak originated in Iowa and Nebraska (e.g., <http://www.cdc.gov/parasites/cyclosporiasis/outbreaks/investigation-2013.html>), and *C. cayetanensis* was detected by the FilmArray GI Panel in a faecal sample from Nebraska, 1 week prior to *Cyclospora* being detected by the state using microscopy [59]. *Cyclospora* testing is not routinely ordered, and the outbreak would likely not have been detected had it not been for the trial of the FilmArray system [59].

The Verigene Enteric Pathogens (EP) test (Luminex) was given FDA approval in 2014 and detects bacteria and viruses but not protozoan pathogens [60]. The EasyScreen™ Enteric Parasite Detection Kit (Genetic Signatures, Sydney, Australia) is a multiplex qPCR kit that detects *G. duodenalis*, *Cryptosporidium* spp., *Entamoeba complex*, *D. fragilis* and *Blastocystis* spp. It utilises bisulfite treatment of DNA that converts cytosine residues to uracil, resulting in the conversion of a 4-base pair sequence (A, C, T, G) into a 3-base pair sequence (A, T, G) (3base™ technology). The advantages of the system are that it can result in improved efficiency of multiplex qPCR detection, as there is less competition between different primers, and allows for primers and probes to be designed that have a more similar melting temperature to each other, resulting in increased amplification efficiency [61]. The sensitivity of the assay was 92% for *G. duodenalis*, 100% for *Cryptosporidium*, 92% for *E. histolytica*, 92% for *D. fragilis*, and 96% for *Blastocystis* [61]. The cost of the assay (excluding labour) is approximately AUD\$20 (~US\$15; including consumables, extraction and amplification controls) [62]. The system can be partially automated using their GS1 Automation System which conducts nucleic acid extraction and setting-up of PCR plates (96- or 384-well format). This reduces hands-on time to ~1 min/specimen. One disadvantage of the assay is that it detects both nonpathogenic and pathogenic *Entamoeba* spp., which requires additional PCR testing to differentiate *E. histolytica* from nonpathogenic species, adding to costs and time required for accurate diagnosis.

Advantages of gastrointestinal panel assays are their broad range capability to detect multiple enteric pathogens and the enhanced sensitivity and specificity of DNA-based detection. This is an important advantage as, currently, testing of faecal samples requires doctors to consider which specific pathogens might be associated with individual cases of diarrhoea and then choose a testing scheme that ensures that all the appropriate pathogens are targeted. Given the diverse array of pathogens associated with diarrhoeal illness, and the diversity of testing methods, it is not surprising that this approach often fails to yield positive results [62]. Another major advantage of enteric panel assays is their high-throughput capacity and their ability to rapidly allow the identification of undiagnosed infections. This ability to save time in detecting a specific infectious organism is an important advantage because it allows specific therapy to be quickly initiated.

A major limitation of these technologies, however, particularly for developing countries, is the costs involved. In addition to the cost of the kits and labour, laboratories must also purchase the analysis instruments which cost up to US\$40 000 or more [43,60], and this can be prohibitive for routine clinical testing and pathogen surveillance purposes, even for laboratories in developed countries. Enteric panel tests, however, still have an important role to play in epidemiological surveillance, research, and outbreak investigations. Another more cost-effective option

is to screen individuals with diarrhoea using microscopy and then test all the microscopy-negative samples using a panel assay. However, for many developing countries with limited health care infrastructure, even this approach is unaffordable.

Emerging Diagnostic Tests

Emerging technologies in parasite detection include both new and underdeveloped methods, which are not yet commercially available for enteric parasites, but have the potential to change the status quo and dramatically improve the diagnosis of enteric protozoan parasite infections in humans, particularly in developing countries. Ideally, these technologies should provide accurate results coupled with cost effectiveness, rapidity, scalability, high-throughput capacity, reproducibility, and sensitivity.

Currently one drawback shared by all multiplex PCR panel tests is that each change to existing primers, or each addition of new primer pairs and probes, necessitates re-evaluation of the sensitivity and specificity of the entire tool. As new sequence data and other information on known pathogens become available, specific quantitative PCR (qPCR) primers and probes may need to be altered or added, but revalidation of an entire multiple-pathogen detection assay is difficult and costly [63]. The TaqMan low-density array (TLDA) (ThermoFischers, USA) uses microfluidic cards in which lyophilized primers and probes for each assay are preloaded and dried onto wells. The advantage of the TLDA platform is that it is a closed system incorporating singleplex PCR methodology, which allows new primers and probes to be added without recalibration of the others already incorporated on the cards. The reagents for each assay are preallocated to the reaction wells for ease-of-use, and the sample needs to be added only once. The primers and probes have a long shelf-life (up to 2 years when refrigerated) as they are lyophilised. TLDA has been used to detect infectious disease pathogens (respiratory); however, low sensitivity for some pathogens [64,65], is an issue to be improved prior to implementation of this methodology as a routine screening test. The system has yet to be tested on enteric parasites.

Another disadvantage of targeted, pathogen-specific PCR panels is that they are only able to identify predefined targets. An alternative strategy takes advantage of the increasing availability, speed, and decreasing cost per base of next-generation sequencing (NGS) offered by deep-sequencing platforms [66]. At present, there are two approaches to NGS. The first (untargeted) approach is shotgun metagenomics, which profiles the entire microbial diversity, or pathobiome (pathogenic microbiome), including bacteria, protozoa, helminths, and viruses, simultaneously, in addition to screening antimicrobial resistance [67]. This technique requires the availability of partial or whole reference genomes, which are compared to the shotgun data following quality processing, curation, and assembly of datasets. While this method has the ability to identify mixed microbial infections (correlated to sequence coverage and depth) and novel microbes, until recently, the lack of reference genomes for many of the most important enteric parasites has limited its use. However, with the increasing availability of enteric parasite genomes, including *Cryptosporidium* [68,69], *Giardia* [70,71], *C. cayetanensis* [72], *E. histolytica* [73], and *Blastocystis* [74,75], shotgun NGS will increasingly be used to identify and develop novel target loci for enteric parasites, particularly those for which previously only limited diagnostic molecular markers were available [76].

The second (targeted) approach to NGS is metabarcoding, which targets predefined domains using universally designed primers. This technique can target small ribosomal subunit (18S) genes amplified from human faecal DNA, while 16S ribosomal genes are utilised for bacterial identification [77,78]. Although NGS has increased sensitivity and is well suited to the amplification of degraded or low-copy-number specimens, inherent errors associated with sequencing homopolymers (i.e., repeats of the same base, such as AAAA), can result in false insertions

and deletions (INDELs) and artefact sequences, and the short sequences generated by many NGS platforms can make it difficult to identify a read to species level [78]. The rate of these errors, however, is platform-specific and has been previously reviewed [79].

While labour time of NGS remains comparable to other molecular diagnostic techniques (e.g., Sanger sequencing), the total turn-around time and cost per sample is still excessive for routine diagnostics and ranges between AUD\$9.50 and AUD\$13.70, and over US\$250, for meta-barcoding and shotgun metagenomics, respectively, without the initial costs associated with purchasing an NGS platform [67,78]. Nonetheless, NGS provides a high-throughput approach to enteric parasite diagnosis, with the ability to detect mixed infections, and in the future it may offer a greater understanding of the correlation between symptomology and diagnosis, and could result in targeted personalised treatments [67]. Another advantage of NGS, at least for bacterial pathogens, is streamlining the workflow in clinical microbiology laboratories by combining pathogen detection, serotyping, virulence and antimicrobial resistance typing into one analysis [80].

Limitations of nucleic-acid-based detection assays in general include (i) technical problems (i.e., amplification inhibition, cross-sample contamination, limited portability etc.), (ii) reagent and labour costs, (iii) limited multiplexing capacity, and (iv) relatively long turnaround time (especially for DNA extraction). Previously, ELISAs were considered the gold standard for parasite detection but suffer from lack of sensitivity and specificity (Table 1). Other protein-based molecular assays are currently being developed, and advances in mass spectrometry (MS) offer potential for improved diagnosis of enteric parasites.

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) is currently being used for rapid and reproducible identification of bacteria, viruses, and fungi in clinical microbiological laboratories [81], and the use of MALDI-TOF MS detection of *Giardia*, *Cryptosporidium*, *Entamoeba*, and *Blastocystis* has recently been reviewed [82]. The technique involves lysis of the organisms to be analysed and ionising biological molecules such as intact proteins [83], which are then accelerated at a fixed potential through a tube with fixed length. A detector records the time of flight (TOF) of the molecular ions measured. A mass analyser that separates ions according to their mass-to-charge ratio (m/z), generates a characteristic spectrum, unique for a given biomolecule, which is then compared against a database of profiles from known organisms [63,81]. The technique is rapid and accurate, does not require highly trained laboratory personnel, is not constrained by sample size and contamination by the host proteins, and is relatively inexpensive, but the initial cost of the equipment is high [63,82]. The application of MALDI-TOF MS to the detection of enteric protozoa has lagged behind other organisms and has been hampered by a number of factors, including cultivation requirements, detection limits, lysis requirements, and the complex biological nature of enteric parasite life-cycle stages present in faecal samples (oo/cysts/trophozoites etc.) [82]. The application of microfluidics technology to MALDI-TOF MS has been shown to increase sensitivity [84] and will increase affordability and improve the detection of enteric parasites.

Despite their high sensitivity and specificity, PCR-based assays are the least feasible to perform at point-of-care (POC) settings due to the relatively high costs (\$3000–\$10 000) associated with thermal cyclers [85]. In addition, PCR is prone to contamination, the nucleic acids must be extracted and purified from the patient sample, and reagents must usually be stored cold to maintain their function [85]. Isothermal amplification techniques, including loop-mediated amplification (LAMP), rolling circle amplification, strand-displacement amplification, and recombinase polymerase amplification (RPA), operate at a single temperature, eliminating the need for a thermocycler, enabling them to be conducted on simple and portable heating systems [86]. Of these, RPA has the advantage of being able to be conducted at room temperature and the

reaction enzymes are stable in dried formulation and can be safely stored without refrigeration for POC use for up to a year [86]. The recent development of an isothermal multiplex RPA assay with lateral flow readout, that is capable of simultaneously detecting and differentiating DNA from *Giardia*, *Cryptosporidium*, and *Entamoeba*, is an important advance in POC detection of enteric parasites [87]. DNA extraction from stool samples at POC remains a challenge but field-deployable DNA extraction devices, that do not require electricity, have been developed [88] and could be designed to process fresh stool samples at POC.

Biosensors represent a promising new class of parasite-detection tools that are under development and have the potential to deliver rapid, accurate, and affordable diagnosis of enteric parasites (due to advances in nanofabrication technologies, microfluidics, and robotics for sample preparation and processing) [82]. A biosensor is usually comprised of specific DNA/proteins (antibodies, enzymes etc.) immobilised on a transducer. The DNA/proteins recognise and bind to specific targets (e.g., on/in enteric parasites) and this molecular recognition is converted, via a transducer, into a measurable signal of various types (electrochemical, optical, mechanical etc.) [89]. The specificity and sensitivity of the detection is determined by the affinity of the DNA or protein for the target on the pathogen of interest. The merger of biosensors with microfluidics, which provides the ability to analyse small sample volumes, thereby minimising costly reagent consumption, offers new promises for POC detection of enteric parasites, including short assay times, low energy consumption, high-throughput analysis, portability, multiplexing ability, and disposability [90]. Despite clinical need, translation of biosensors from research laboratories to clinical detection has remained limited, although increasingly they are being developed for enteric parasites [91,92]. Challenges to be overcome include sample preparation, system integration (all components of the assay in one device) and the fact that the complex nature of faecal samples can lead to nonspecific binding and aberrant signals [93].

The development of sophisticated synthetic polymers that can be used to interact with high specificity with target pathogens is another technology that holds promise for the future, particularly when coupled with improvements in the sensitivity of analytical devices, for example, using techniques such as surface-enhanced resonance Raman scattering (SERRS), are enabling the detection of biopolymers at the attomole level and below [94]. The application of this technology to enteric protozoans is in its infancy [95,96] and future work is necessary to investigate the kinetics of adhesion, the influence of different solution conditions etc., and the successful merging of polymer detection with SERRS.

Diagnostic Challenges in Resource-Poor Countries

For developing countries, diagnostics need to be low cost, require minimal or no external power, be able to be run on portable and easy-to-maintain equipment, be usable without extensive training, not require refrigerated reagent storage, and deliver accurate and unambiguous results rapidly [85]. The WHO has established a set of principles to guide the development of diagnostics for these low-resource, POC settings known as ASSURED. Ideally, diagnostics for enteric parasites should be Affordable, Sensitive, Specific, User-friendly, Rapid and Robust, Equipment-free, and Deliverable to end-users.

The development, production, and marketing of a new diagnostic assay, however, is a complex and expensive undertaking, particularly for use in developing countries, and funds to establish manufacturing capacity or networks for distribution and maintenance in developing countries are often more difficult to source [97]. Poorly defined markets and low expectation of return on investment impede access to finance from commercial sources, and public or philanthropic funding are often essential (e.g., http://www.who.int/publications/Increasing_Access_to_Diagnostics_Through_Technology_Transfer.pdf?ua=1). Barriers to the successful implementation of POC diagnostics in resource-limited settings

include profitability, issues with intellectual property, lack of feedback, and engagement with end-users before tests are designed and research and development begins, transition from experimental trials at academic level, to clinical validation and the need to educate health care workers in proper test usage and the benefits of the test. For example, it is not uncommon for devices that perform well in a controlled environment to fail when used in tropical regions, and therefore optimisation studies using prototype assays at sites of intended use should be undertaken early in product development to ensure that new diagnostic tests are able to withstand exposure to extremes of temperature during transport and storage [97]. In addition, quality assurance and maintenance of equipment is often difficult to achieve in developing countries, particularly for devices used outside of the laboratory network and at the POC [97].

Concluding Remarks

Improved diagnosis of enteric parasitic diseases will contribute to a better understanding of complex and severe clinical cases, enhance infection control efforts, reduce overall social and healthcare costs, and improve treatment outcomes in both developed and developing countries [36]. Many questions still remain (see Outstanding Questions). As techniques continue to advance, more and more pathogens can be detected simultaneously from faecal samples. Which of these pathogens are responsible for diarrhoea in the individual being tested, and which are responsible for asymptomatic infection, requires further investigation. For example, screening of faecal samples using enteric panels has identified higher than expected levels of mixed infections [43,57], and suggests that the presence of multiple pathogens in diarrheal stool samples may be underestimated by current routine tests. This challenges the current paradigm that one pathogen is responsible for a particular diarrhoeal disease episode and suggests the possibility that multiple organisms can contribute to diarrhoea. However, the detection of a DNA sequence does not indicate the presence of a viable organism, and many enteric parasites and bacteria can exist asymptotically [98–101]. Further research is essential to clarify the putative role of mixed infections in the development of diarrhoeal disease. It is also important to note that the detection of an asymptomatic infection may still be of clinical relevance as asymptomatic enteric parasite infections can lead to irritable bowel syndrome and chronic fatigue [102,103], and in children can lead to growth stunting [104–106]. Asymptomatic infections are also associated with the spread of disease and prolongation of outbreaks due to silent transmission [101,107,108]. Doctors will have to assess the clinical importance of mixed infections, and new algorithms need to be developed to assist doctors in interpreting the relationship between pathogen detection and occurrence of diarrhoea and other clinical symptoms, particularly when mixed infections are detected. This will likely lead to a better understanding of complex clinical scenarios and will allow more efficient treatment regimens that reduce secondary infections and failed treatments [35] and avoid the overuse of drugs and/or antibiotics. Increased detection of mixed infections is also particularly relevant in light of the growing awareness of the importance of the human gut microbiome and the role of the gut microbial community in the development of enteric diseases [109–112]. Future studies should consider the 'gut-ecosystem' and concentrate on the contribution of multiple pathogens to individual cases of diarrhoea using untargeted approaches like NGS, as the presence of one pathogen may favour other opportunistic infections and therefore it is possible that the co-occurrence of multiple pathogens may be correlated. Further research into the interactions between host–parasite–microbiota and their outcomes, and the administration of probiotics as possible therapeutic agents to control the proliferation of intestinal pathogens, is essential [111]. The advent of real-time sequencing of enteric parasites and the ability to perform real-time outbreak investigation will also make a significant impact on public health [63].

Commercialisation is a crucial step in the development of new parasite detection methods for POC diagnostics, but is not always driven by the socioeconomic benefit brought by the

Outstanding Questions

Can emerging technologies produce low cost and accurate detection assays under field conditions?

What is the prevalence and clinical significance of mixed infections?

Can the co-occurrence of multiple pathogens be correlated?

What is the clinical significance of 'asymptomatic' infections?

What is the role of the gut microbial community in the development of enteric diseases?

If links between pathogen-presence and core microbiota-perturbations are found, can this be used as a biological indicator, for example, risk factor for disease?

Can probiotics be used to treat enteric parasites?

technology. Ultimately, the successful development of POC tests for enteric parasites in developing countries relies on building successful partnerships with both the scientists and health care systems in these countries.

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