‘A comparative study of traditional and molecular diagnostic methods for detection of gastrointestinal parasites in Nepalese migrants to the UK’Running Title: Comparing diagnostic methods for parasites.

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Summary

Background

We evaluated the results of examining a single faecal sample for gastrointestinal parasites (GIP) using a combination of traditional methods with multiplex qPCR for helminths and protozoa, compared to a reference standard of examining three faecal samples from each person using traditional diagnostic methods alone.

Methods

Three faecal samples were collected at weekly intervals from 596 healthy Nepalese men. Each sample underwent formalin-ethyl acetate (FEA) concentration and light microscopy, and charcoal culture. The combined results of these investigations for all three stool samples were designated the reference standard. The first sample was also analysed using a multiplex TaqMan™ qPCR assay, screening for five helminths and three protozoa. We compared sensitivity and specificity of analysing the first faecal sample with qPCR alone, or a hybrid approach combining qPCR with traditional methods, to the reference standard. Additionally, a serum sample was taken from each participant for *Strongyloides stercoralis* IgG ELISA.

Results

The reference standard identified 139 GIP infections in 133 (22.3%) participants. Use of qPCR alone in one stool identified 176 infections in 147 (24.8%) participants, rising to 187 infections in 156 (26.3%) when combined with FEA microscopy and charcoal culture. The sensitivity of this latter hybrid approach was 100% for *Strongyloides* spp., 90.9% for *Trichuris trichiura*, 86.8% for hookworm species and 75% for *Giardia duodenalis* compared to the

reference standard. The hybrid approach increased the detected cases of *G. duodenalis* by 4.5% (46 cases) overall, *T. trichiura* by 2.9% (18 cases), *Strongyloides* spp. by 1% (6 cases), and hookworm by 0.5% (8 cases), compared to the reference standard.

Conclusion

Examination of a single faecal sample using qPCR alone showed superior or equivalent sensitivity to traditional methods for most GIP infections when both were compared to the reference standard. Combining molecular and traditional methods to analyse a single stool improved the detection rate for most studied parasites. This approach has value in settings where repeated sampling and/or faecal culture for helminths is impractical, but molecular diagnostics are available.

Keywords

Migrant; Nepal; United Kingdom; parasite; qPCR; diagnostics

Introduction

Infections with gastrointestinal parasites (GIP) remain an important global health problem, affecting some of the world's poorest communities. It has been estimated that around a quarter of the global population are infected by soil-transmitted helminths (STH) [1, 2]. The burden of *Strongyloides stercoralis*, recently added to the World Health Organisation STH control strategy [3], is thought to have been previously underestimated, with recent data suggesting 300-600 million people may be infected [4, 5]. Despite international efforts, STH were still estimated to cause two million lost DALY's as of 2019, and millions of people are affected by protozoa such as *Giardia duodenalis, Cryptosoporidium* spp. and *Entamoeba histolytica* [6-8].

With increased international travel and globalisation, there is a need to improve diagnosis of GIP in non-endemic countries for migrant populations and returning travellers. This is complicated by the possibility of low intensity infections which may lack clinical symptoms and may be harder to detect. The United Kingdom Armed Forces recruits soldiers for its Gurkha regiments directly from Nepal, with around 200-400 personnel arriving in the UK annually. Historically, GIP infection rates have been high in Gurkha recruits [9], and more recent research led to the introduction of a routine screening programme in 2012 [10, 11]. Recently published data from nine years of this

programme report an overall prevalence of GIP of 20.5% [12]. Wider data on the prevalence of GIP infection in Nepal are heterogenous with very high prevalences of around 25-30% in some studies, while others report relatively low prevalences around 1-2% [13-16]. To our knowledge, the routine screening of Nepalese Gurkha recruits represents one of the longest running screening programmes for GIP in an immigrant population from an endemic area to the UK.

Whilst GIP infection intensity has never been formally quantified in the Gurkha recruit population, it has generally been noted to be low [12]. Low intensity infections present a challenge for diagnosis even for specialist parasitology reference laboratories. Traditional copro-microscopic methods have variable sensitivity and require an experienced operator to provide accurate results [17, 18]. To overcome this, it has been recommended that more than one stool sample per-patient be analysed in order to improve diagnostic sensitivity [19]. However, the strategy of screening multiple stools presents logistical issues and requires additional laboratory resources and time. A potential solution to improve the sensitivity of examining a single stool would be to incorporate a molecular assay such as qPCR for the detection of GIP, as multiple studies have reported improved sensitivity over more traditional microscopy-based approaches [20-26].

This study compared sensitivity and specificity of a multiplex helminth and protozoa qPCR alone and in different combinations with traditional diagnostic approaches in a single faecal sample, with a proposed reference standard analysis of three consecutive stools using traditional faecal methods, in the Nepalese Gurkha recruit population.

Materials and methods

Sample collection

Nepalese Gurkha recruits arriving in the UK have been screened since 2012 for faecal parasites [12]. We re-examined stored faecal samples with multiplex qPCR from the cohorts arriving 2014-2016 inclusive. All 596 participants were males aged 18-21 who had arrived in the UK within 14 days prior to providing a single serum sample and three faecal samples at weekly intervals. All participants had recently undergone a rigorous military selection process and were in presumed good physical health. Samples were collected as follows;

1. Week 1: Serum sample and stool sample 1 collected.
2. Week 2: Stool sample 2 collected.
3. Week 3: Stool sample 3 collected.

The schedule of sample collection is also shown in Figure 1. All samples were couriered at ambient temperature to the Clinical Diagnostic Parasitology Laboratory (CDPL) at Liverpool School of Tropical Medicine (LSTM) as soon as they were collected, typically within 18-24 hours of passage. No preservatives or buffers such as sodium acetate-acetic acid-formalin (SAF) were added to faecal samples. Upon receipt at LSTM, the samples were stored at room temperature and processed within 48 hours. Faecal material from stool sample one was frozen at -20^{o}C without preservatives for later DNA analysis. The following tests were performed on samples as outlined below.

Formalin-ethyl acetate (FEA) concentration and light microscopy

A faecal concentration was performed on all samples using a modification of the Ritchie formalin-ether method [27] utilising the Evergreen FPC ® system as discussed in Nevin et al [12]. Slides were examined using light microscopy by two experienced microscopists at x100 and x400 magnification and any pathogenic and non-pathogenic parasitic organisms were recorded. Slides were also stained using the modified Ziehl Neilson technique to identify *Cryptosporidium* spp. or other coccidia, and iodine was used as an aid to light microscopy according to microscopist judgement, but no other staining process was routinely undertaken. The presence of *Entamoeba* cysts was recorded; however, it is not possible to make a distinction microscopically between pathogenic *E. histolytica* and species which are non-pathogenic or have disputed pathogenicity (*Entamoeba dispar*, *moshkovskii or bangladeshi*). We have referred to this morphologically identical group as *Entamoeba* spp. throughout the text. This does not include morphologically distinct species usually considered to be non-pathogenic such as *Entamoeba coli* and *Entamoeba hartmanni*.

Charcoal culture

Charcoal culture was performed on all stool samples. Culture allows rhabditiform larvae in the sample to employ a free-living cycle in which they develop into free living adult stages, which in turn give rise to filariform larvae easily seen using microscopy. Increased sensitivity can be achieved over microscopy after faecal concentration because of the large volume of faecal material used and the amplification of worm numbers due to the free-living stage. The culture method was modified from the protocol described by Dancescu [28] as described by Nevin et al [12]. Following incubation, the faecal mixture and water were examined for helminth larvae using x30 magnification. Larvae were differentiated morphologically between *Strongyloides* spp. and hookworm spp. based on buccal cavity size for L1

larvae (short for *Strongyloides* vs long for hookworm) and tail-end shape for L3 larvae (forked for *Strongyloides* vs pointed for hookworm). Whilst differentiation between hookworm species is possible on L3 infective larvae [29], this was not routinely done as clinical laboratory practice.

DNA extraction and TaqMan™ qPCR

The stored first stool sample from each of the 596 participants, underwent DNA extraction and molecular analysis as described by Cunningham et al [30]. In brief, archived frozen faecal samples were allowed to defrost at room temperature after which 0.1 mg of faeces were removed and added to a 2 ml screwcap tube containing 0.9 mg of 1.4 mm ceramic beads (Qiagen, Manchester, UK). To this the PVPP DNA (Merck, Gillingham, UK) extraction buffer was added, and the sample was frozen overnight at -20ºC. The following day the samples were allowed to defrost and underwent a bead-beating step, at 3000 rpm for 30 seconds using the MagnaLyser (Roche, Burgess Hill, UK) system. Following bead-beating, samples were processed using the QIAmp DNA Mini kit with the following modifications to the protocol: i) 1 µL of phocine herpes virus-1 (PhHV-1), per-sample, was added to the AL buffer, to act as an internal positive control and ii) the DNA was eluted in 100 µL of AE buffer.

The faecal samples were screened using a two-tube qPCR assay, with Reaction 1 screening for *G. duodenalis, Cryptosporidium* spp*., E. histolytica* and the PhHV-1 internal positive control. It should be noted no other *Entamoeba* species was included in the assay. Reaction 2 screened for *A. lumbricoides, T. trichiura, Strongyloides* spp*., Schistosoma* spp. and hookworm (both *Ancylostoma duodenale* and *Necator americanus*). These two assays used pre-published primers and probes [26, 31-36] and were optimised and carried out in 20µL reactions on the Rotor-Gene Q platform (Qiagen, Manchester, UK) (Appendix 1). For a single 20µL, 2µL of DNA template and 10µL of Bio Rad iQ™ Supermix (Bio-Rad, Watford, UK) was used. The concentrations of the primers and probes are given in Appendix 1, with the remaining volume being made of nuclease free water. The thermocycler conditions were as follows: hold at 95ºC for 15 minutes; then 50 cycles of 94ºC for 15 seconds, followed by 60ºC for 60 seconds. A quantification cycle (C_q) value of <38 was considered positive for our analyses.

Serological analysis

All serum samples had an in-house antibody ELISA performed to detect IgG antibodies to *S. stercoralis*. Antigen for this ELISA was obtained from pooled L3 *S. stercoralis* larvae collected from patient samples at the CDPL at LSTM as

described by Nevin et al [12]. The assay was validated using known positives, known negatives, known cross-reactive samples and accredited by a national laboratory evaluation scheme.

Statistical analysis

Positive diagnostic tests in stool samples were summarised using frequencies and percentages. The cumulative yield of GIP detected in two stools and three stools was obtained. The summary yield of FEA concentration and light microscopy, plus charcoal culture of all three stools was considered as the reference standard test for each parasite and is referred to as such throughout the text. We compared the reference standard to the following index tests on the first stool sample;

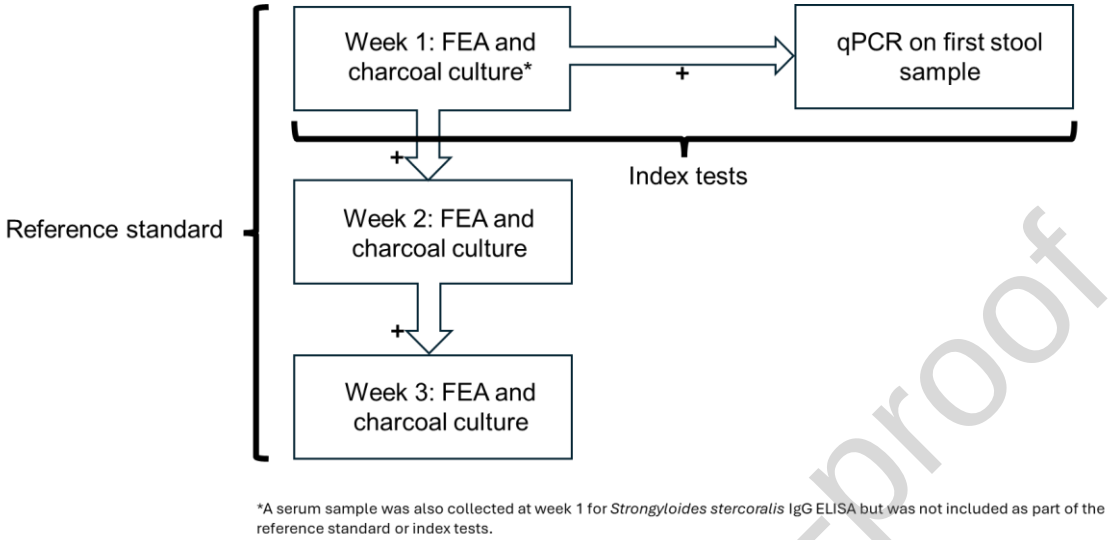
i. qPCR alone
ii. qPCR combined with FEA concentration and microscopy
iii. qPCR combined with FEA concentration and microscopy plus charcoal culture (for *Strongyloides* and hookworm spp.).

Sensitivity and specificity, and their corresponding 95% confidence intervals (CI) were estimated to compare results of tests on the first stool sample with the suggested reference standard. Fleiss' kappa (κ) was performed to quantify agreement between the reference standard and other test combinations. Poor agreement was defined as a kappa value of <0.00, slight agreement as 0.00-0.20, fair agreement as 0.21-0.40, moderated agreement as 0.41-0.60, substantial agreement as 0.61-0.80, and near perfect agreement as 0.81-1.00.

The schedules of sample collection for the reference standard and hybrid approaches are shown in Figure 1. Whilst the reference standard only included faecal examination methods, *S. stercoralis* IgG ELISA was performed and findings are briefly discussed in the Results section and can be found in Supplementary Table 5 and Supplementary Table 6 in Appendix 2. Analysis was conducted for each parasite separately using SAS software version 9.4 and GraphPad Prism v10, and all tests were performed at 5% significance level.

Fig 1. Flow diagram of sample collection from participants. Stool samples were collected at weeks one, two and three. Each stool sample had FEA concentration and microscopy, and a charcoal culture performed. Combined

results from FEA concentration and microscopy, and charcoal culture on three stools was designated the reference standard. qPCR was performed on the first stool sample only. The index tests were qPCR alone, qPCR combined with FEA concentration and microscopy, and qPCR combined with FEA concentration and microscopy plus charcoal culture.



*A serum sample was also collected at week 1 for *Strongyloides stercoralis* IgG ELISA but was not included as part of the reference standard or index tests.

Ethics and Funding

Samples were collected on a clinical basis as part of a routine medical examination for military service, and verbal consent was obtained from each participant prior to sample collection. The qPCR assay screened for pathogens as detailed in the established contract for clinical tests established with the Ministry of Defence and as such no additional ethical approval was required. Any additional positive results yielded by qPCR were discussed with specialist clinicians and follow-up and treatment was offered to individuals as appropriate.

Results

The numbers of positive individuals and percentage prevalence for each GIP infection are shown in Table 1. Results are given for FEA concentration and light microscopy, and charcoal culture for either the first, second or third stool sample, as well as cumulative results. Table 1 also shows the number of positive individuals and the percentage prevalence for each GIP from the first stool sample after undergoing multiplex qPCR assay. The qPCR PhHV-1 internal positive control failed for 3/596 samples (all of which were negative using the reference standard). These samples were excluded from comparison analyses.

Table 1. Number of positive individuals and prevalence of GIP per stool sample, including cumulative positive individuals and prevalences. The results are split between FEA concentration and light microscopy, charcoal culture, combination of FEA and culture (reference standard) and the results of the first stool sample screened using qPCR, alone or combined with traditional methods. New individuals identified as positive per stool 1, 2, or 3 are denoted by *n* and the cumulative number of positive individuals is denoted by (*n*). The percentage of individuals positive and cumulative percentages are denoted by *n* and (*n)* respectively under the heading % prevalence. Faecal cultures were only performed for diagnosis of *Strongyloides* spp. and hookworms.

		Diagnostic test								
		FEA concentration and microscopy			Charcoal culture			FEA + charcoal culture	qPCR	qPCR + FEA + charcoal culture
		Stool 1	Stool 2	Stool 3	Stool 1	Stool 2	Stool 3	All 3 stools (reference standard)	Stool 1 only (index tests)	
Strongyloides spp.	n=positive (cumulative)	0	0	0	2	0 (2)	1 (3)	3	8	9

	% prevalence (cumulative)	0.0	0.0	0.0	0.3	0.0 (0.3)	0.2 (0.5)	0.5	1.3	1.5
Hookworm spp.	n=positive (cumulative)	7	8 (15)	2 (17)	17	12 (29)	8 (37)	38	39	41
	% prevalence (cumulative)	1.2	1.3 (2.5)	0.3 (2.9)	2.9	2.0 (4.9)	1.3 (6.2)	6.4	6.5	6.9
Schistosoma spp.	n=positive (cumulative)	0	0	0				0	1	1
	% prevalence (cumulative)	0.0	0.0	0.0				0.0	0.2	0.2
Ascaris lumbricoides	n=positive (cumulative)	1	0 (1)	0 (1)				1	1	1
	% prevalence (cumulative)	0.2	0.0 (0.2)	0.0 (0.2)				0.2	0.2	0.2
Trichuris trichiura	n=positive (cumulative)	4	5 (9)	2 (11)				11	28	28
	% prevalence (cumulative)	0.7	0.8 (1.5)	0.3 (1.8)				1.8	4.7	4.7
Giardia duodenalis	n=positive (cumulative))	40	23 (63)	13 (76)				76	99	103
	% prevalence (cumulative)	6.7	3.9 (10.6)	2.2 (12.8)				12.8	16.6	17.3
Entamoeba spp.	n=positive (cumulative)	4	4 (8)	2 (10)				10	0	4
	% prevalence (cumulative)	0.7	0.7 (1.4)	0.3 (1.7)				1.7	0.0	0.7

Using the combination of FEA concentration and microscopy plus charcoal culture on three stools (reference standard) a total of 139 GIP infections were identified in 133 (22.3%) individuals. In comparison, the qPCR assay performed on the first stool identified 176 GIP infections in 147 (24.8%) individuals. The number of single infections, and mixed infections, using the reference standard approach was 127 (21.3%) single infections and 6 (1.0%) mixed infections. The qPCR assay identified 122 (20.6%) single infections and 25 mixed infections (4.2%).

Sensitivities of the qPCR assay against the proposed reference standard for each GIP are shown in Table 2 and Table 3. Sensitivity of qPCR ranged from 100.0% (95% CI 2.50-100.00%) for *A. lumbricoides* to 0.0% (95% CI 0.00-30.85%) for *Entamoeba* spp*.* The specificity ranged from 100% (95% CI 99.37-100.0%) for *A. lumbricoides* and *Entamoeba* spp. to 91.2% (95% CI 88.38-93.45%) for *G. duodenalis*. Full specificity data can be found in Supplementary Table 1 in Appendix 2.

Fleiss' kappa demonstrated fair agreement between qPCR and reference standard for *Strongyloides* spp. (κ = 0.36), moderate agreement for *T. trichiura* and *G. duodenalis* (κ = 0.50 and κ = 0.54 respectively), substantial agreement for hookworm spp. (κ = 0.79) and perfect agreement for *A. lumbricoides* (κ = 1.00). Values for kappa calculations, standard error of κ and 95% CI can be found in Supplementary table 1 in Appendix 2.

Comparisons of sensitivities and prevalence using a hybrid approach (qPCR, plus FEA concentration and microscopy, plus charcoal culture on the first stool sample) versus the reference standard are shown in Table 2 and Table 3. Sensitivity ranged from 100% for *Strongyloides* and *A. lumbricoides* (95% CI 29.24-100.00%, and 2.50-100.00% respectively) to 40.0% (95% CI 12.16-73.76%) for *Entamoeba* spp. Specificities ranged from 100.0% (95% CI 99.37-100.00%) for *Entamoeba* spp. to 91.2% for *G. lamblia* (95% CI 88.38-93.45%). Full specificity data can be found in Supplementary Table 2 in Appendix 2.

Fleiss' kappa demonstrated moderate agreement between the hybrid approach and reference standard for *Strongyloides* spp., *T. trichiura*, *G. duodenalis* and *Entamoeba* spp. (κ = 0.50, κ = 0.50, κ = 0.57, κ = 0.57 respectively), and near perfect agreement for hookworm spp. and *A. lumbricoides* (κ = 0.82 and κ = 1.00 respectively). Values for kappa calculations, standard error of κ and 95% CI can be found in Supplementary table 2 in Appendix 2.

Table 2. Comparison of prevalences obtained and sensitivities of different combinations of methods for detection of *Strongyloides* and hookworm spp. against the reference standard of FEA concentration and microscopy plus charcoal culture of three stools.

		Diagnostic tests					
		FEA and microscopy	FEA microscopy + culture	qPCR alone	qPCR + FEA and microscopy	qPCR + FEA and microscopy + charcoal culture	Reference standard
		Stool 1	Stool 1	Stool 1	Stool 1	Stool 1	3 Stools
Strongyloides spp.	Prevalence (%)	0.0 (0.00-0.77)	0.3 (0.00-1.30)	1.3 (0.63-2.67)	1.3 (0.63-2.67)	1.5 (0.75-2.89)	0.5 (0.10-1.54)
	Sensitivity (%)	0.0 (0.00-70.76)	66.7 (9.43-99.16)	66.7 (9.43-99.16)	66.7 (9.43-99.16)	100.0 (29.24-100.00)	N/A
Hookworm spp.	Prevalence (%)	1.2 (0.52-2.46)	3.5 (2.29-5.36)	6.5 (4.80-8.84)	6.5 (4.80-8.84)	6.9 (5.09-9.22)	6.4 (4.66-9.51)
	Sensitivity (%)	18.4 (7.74-34.33)	55.2 (38.30-71.38)	81.6 (65.67-92.26)	81.6 (65.67-92.26)	86.8 (71.91-95.59)	N/A

Includes reference standard versus the following index tests on the first stool only:

i. FEA concentration and microscopy.

ii. FEA concentration and microscopy plus charcoal culture.

iii. qPCR alone.

iv. qPCR plus FEA concentration and microscopy.

v. qPCR plus FEA concentration and microscopy, plus charcoal culture.

Only those pathogens which have potential to be diagnosed via charcoal culture (i.e. *Strongyloides* spp. and hookworm spp.) are shown in this table. Specificity values, and values for calculation of sensitivity and specificity can be found in the supplementary tables in Appendix 2. Ninety-five percent CI are given in brackets.

Table 3. Comparison of prevalences obtained and sensitivities of different combinations of methods for detection of gastrointestinal parasites against the reference standard of FEA concentration and microscopy plus charcoal culture of three stools.

		Diagnostic tests			
		FEA and microscopy	qPCR alone	qPCR + FEA and microscopy	Reference standard
		Stool 1	Stool 1	Stool 1	3 Stools
Schistosoma spp.	Prevalence (%)	0.0 (0.00-0.77)	0.2 (0.00-1.04)	0.2 (0.00-1.04)	0.0 (0.00-1.04)
	Sensitivity (%)	N/A	N/A	N/A	N/A
Ascaris lumbricoides	Prevalence (%)	0.2 (0.00-1.04)	0.2 (0.00-1.04)	0.2 (0.00-1.04)	0.2 (0.00-1.04)
	Sensitivity (%)	100.0 (2.50-100.00)	100.0 (2.50-100.00)	100.0 (2.50-100.00)	N/A
Trichuris trichiura	Prevalence (%)	0.7 (0.20-1.78)	4.7 (3.25-6.73)	4.7 (3.25-6.73)	1.8 (0.99-3.32)
	Sensitivity (%)	36.4 (10.93-69.21)	90.9 (58.72-99.77)	90.9 (58.72-99.77)	N/A
Giardia duodenalis	Prevalence (%)	6.7 (4.95-9.03)	16.6 (13.83-19.82)	17.3 (14.45-20.53)	12.8 (10.30-15.68)
	Sensitivity (%)	52.6 (40.84-64.21)	69.7 (58.13-79.75)	75.0 (63.74-84.23)	N/A
Entamoeba spp.	Prevalence (%)	0.7 (0.20-1.78)	0.0 (0.00-0.77)	0.7 (0.20-1.78)	1.7 (0.87-3.10)
	Sensitivity (%)	40.0 (12.16-73.76)	0.0 (0.00-30.85)	40.0 (12.16-73.76)	N/A

Includes reference standard versus the following index tests using only the first stool sample:

i. FEA concentration and microscopy alone.

ii. qPCR alone.

iii. qPCR plus FEA concentration and microscopy.

This table covers pathogens diagnosed without the use of charcoal culture. No individuals were positive for *Cryptosporidium* either using FEA concentration or qPCR. Specificity values, and values for calculation of sensitivity and specificity can be found in the supplementary tables in Appendix 2. Ninety-five percent CI are given in brackets.

In routine practice in many microbiology laboratories, specialist parasitological techniques such as charcoal culture are not performed, and the logistic burden involved with acquiring multiple stool samples may result in only one sample being processed. Table 2 and Table 3 summarise the sensitivity of examination of a single stool sample using different combinations of methods, compared to the reference standard. With the exception of *Entamoeba* spp. and *A. lumbricoides*, adding qPCR to the diagnostic panel increased sensitivity compared to FEA concentration and microscopy alone. Sensitivity using FEA concentration and microscopy alone for hookworm was 18.4% (95% CI 7.74-34.33%), rising to 81.6% (95% CI 65.67-92.26%) when qPCR was added. For *Strongyloides,* sensitivity rose from 0.0% (95 % CI 0.00-70.76%) to 66.7% (95% CI 9.43-99.16%), for *G. duodenalis* from 52.6% (95% CI 40.84-64.21) to 75.0% (95% CI 63.74-84.23%) and for *T. trichiura* from 36.4% (95% CI 10.93-69.21%) to 90.9% (58.72-99.77%). Except for *Entamoeba* spp., a hybrid qPCR/traditional diagnostic approach on one stool yielded the same or higher prevalence of infection than the reference standard.

Serological testing of samples for *S. stercoralis* by IgG ELISA was positive in 31 (5.2%, 95% CI 3.67-7.31%) individuals. Adding the reference standard diagnostic methods brought the number of individuals with a positive test for *Strongyloides* to 34 (5.7%, 95 CI 4.09-7.89%). Of the 31 seropositive individuals, two were positive on qPCR, and none were positive via the reference standard tests. Combining all methodologies for *Strongyloides* diagnosis (IgG ELISA, reference standard and qPCR) gave a total of 38 positives (6.4%, 95% CI 4.66-8.65%). Details of *Strongyloides* diagnostic tests can be found in Supplementary Table 5 and Supplementary Table 6 in Appendix 2.

Discussion

In this study we aimed to compare the sensitivity and specificity of analysing a single stool sample using qPCR in parallel with traditional diagnostic methods (FEA concentration and microscopy, and charcoal culture) with a reference standard of three stool samples which each underwent FEA concentration and microscopy plus charcoal culture. The population studied included healthy young men without overt symptoms or recent treatment for gastrointestinal parasites. We demonstrate a multiplex qPCR had equivalent or superior sensitivity compared to microscopy (and charcoal culture for *Strongyloides* and hookworm spp.) of a single faecal sample.

There was considerable variation in the sensitivity of the qPCR and the single stool approach compared with the suggested reference standard for the diagnosis of protozoa. The only pathogenic or potentially pathogenic protozoa detected were *G. duodenalis* and *Entamoeba* spp.*;* no *Cryptosporidium* spp. were detected by any method.

For *G. duodenalis*, a combined hybrid single stool approach had a sensitivity of 75% compared with the reference standard. However, the addition of the qPCR resulted in the detection of further 46 positive cases which had not been identified using FEA concentration and microscopy on a single stool. We found a higher prevalence with both qPCR alone (16.6%) and the combined approach (17.3%) compared to the reference standard (12.8%). This is consistent with previous studies, which have described PCR as a similarly sensitive or more sensitive diagnostic test for protozoa compared with microscopy, although these studies often compare a single stool sample [20, 23-25, 37, 38]. For example, in one study using immunofluorescence assay (IFA) as the reference standard, PCR had a sensitivity of 91%, and specificity of 95.1%. The same study reported light microscopy to have a sensitivity of 31% versus qPCR [39]. However, increased sensitivity of PCR is not a universal finding: one study compared three different commercial multiplex PCRs for *G. duodenalis,* yielding sensitivities of 89%, 64% and 41% vs microscopy of a single stool [40].

Compared to the reference standard, the detection of *Entamoeba* spp*.* using a hybrid approach had a sensitivity of 40%, with 6/10 cases not detected. There were no positive results on *E. histolytica* qPCR. It is probable that most or all *Entamoeba* cysts observed by microscopy were species of non-pathogenic or disputed pathogenicity species such as *E. dispar, E. moshkovskii or E. bangladeshi,* as these have been more commonly reported in Nepal than pathogenic *E. histolytica* [41]. Microscopy is unable to differentiate between these species, and the multiplex qPCR used in this study only included primers to detect *E. histolytica*. It is therefore likely our study underestimates the sensitivity of a single stool hybrid qPCR/traditional approach for detection of *E. histolytica*.

The use of PCR for clinical diagnosis of helminth infections is less widespread than for protozoa, even in high resource settings such as the UK, and the literature on comparison with traditional diagnostic methods is limited. When comparing total numbers of cases detected, we found qPCR detected a greater number of cases than the reference standard, with the exception of *A. lumbricoides*, where both methodologies only detected a single case (Table 1). However, qPCR did not always detect every positive identified by the reference standard. We found sensitivities of 100%, 90.9%, 81.6%, and 66.7% when comparing qPCR alone against the reference standard for *A. lumbricoides*, *T. trichiura*, hookworm spp. and *Strongyloides* spp. respectively. Barda et al compared PCR to Kato-Katz with broadly similar results (sensitivity of 87.5% for *A. lumbricoides*, 89.1% for *Trichiura* and 72.7% for hookworm), and found single PCR to be as sensitive, or more sensitive than quadruple Kato-Katz [19]. Multiple studies have similarly reported a high sensitivity for PCR compared with traditional methods in a range of helminth infections [22, 26, 42-45].

In keeping with a larger study in this Nepalese population [12], the sensitivities of FEA concentration and microscopy of a single stool to detect hookworm spp. and *Strongyloides* were poor at 18.4% and 0.0% respectively, and addition of charcoal culture improved sensitivities to 55.2% and 66.7% respectively. Addition of qPCR further improved these to 86.8% and 100% respectively, compared to the reference standard using three stools. Sensitivity for *A. lumbricoides* and *T. trichiura* using the hybrid approach was the same as for qPCR alone, at 100% and 90.9% respectively.

Utilising the qPCR platform, *Strongyloides* DNA was detected in an additional six patients who were negative using microscopy and culture. Traditional microscopy and culture techniques are known to be insensitive in *Strongyloides* spp. diagnosis and thus some of these may have represented undetected true positives. In contrast, some authors have reported reduced sensitivity using PCR for diagnosis of *Strongyloides* spp. compared with the Baermann method [46]. However, those studies did not include mechanical bead beating during their DNA extraction, which has been shown to improve diagnostic yield of PCR [47]. In our study, qPCR had fewer positive results compared to serological testing, in keeping with other reports[47]. We found an overall seropositivity for *S. stercoralis* of 5.2% (31/596). However, identifying which of these represents a true positive result is challenging. Serology has been reported to have high sensitivity [29]. However, specificity can be impaired by cross reactions with other helminth

species [48] and raised titres may represent previously resolved infection as serology can remain positive for at least several months [49].

None of the three patients diagnosed with *Strongyloides* spp. via the reference standard approach were serologically positive. Of the eight patients who were qPCR positive, two were seropositive and six negative. The reasons for this are unclear; the in-house IgG ELISA underwent thorough validation and was estimated to have a sensitivity of 98%. Whilst it is possible that some individuals had recent infections and had not yet mounted a serological response, this may not fully explain the lack of seropositivity we found.

Whilst increased sensitivity of PCR compared with traditional methods for diagnosis of helminth infections has been frequently reported, at present this approach is not widely available, particularly in low resource settings [50], due in part to access to expensive equipment for mechanical lysis. The importance of a mechanical bead beating step, as used in our protocol, has been described by Autier et al [47] and by Kaisar et al [51]. This may be particularly relevant when parasite burden is low, as in our population. As materials and equipment become more affordable, qPCR may be able to improve the diagnostic approach in remote, low resource environments by reducing the number of faecal samples required.

Study limitations

The validity of a positive qPCR result representing active infection needs to be considered in the context of a patient's clinical history. For example, in *G. duodenalis* infections DNA can be detected several days post treatment due to the persistence of DNA in the host [52]. Therefore, a positive qPCR may not indicate an active infection, but merely that DNA of the target organism is present. To our knowledge, no participants had received recent treatment for any of the tested conditions. It must also be considered that qPCR may produce false positive results due to cross contamination [53], although the inclusion of quality control measures such as negative extraction controls help address this issue.

In our study we chose a Cq cut of <38 to determine a positive result for further analyses. We acknowledge the possibility that Cq values higher than this may reflect true positive results, and have previously been reported as such [26]. However, we have chosen a more conservative threshold, in part to mitigate contamination risk due to a lack of automation in our laboratory processes. In our laboratory practice, any results with Cq values above this

threshold are reported as 'equivocal', and a clinical assessment including symptomatology, exposure risk and travel history is undertaken to determine the need for treatment or further testing. Combining a positive result with these parameters allows qPCR to play an important role in informing clinical decisions.

The lack of a universally validated true gold standard for diagnosis of GIP is a challenge when attempting to validate any novel diagnostic approach. We used a reference standard of FEA concentration and microscopy, plus charcoal culture, of three stool samples. However, microscopic techniques are highly investigator dependent, with pathogen misidentifications reported in previous literature [54]. To mitigate this, all microscopy was performed by at least two experienced operators. Charcoal culture has been demonstrated to be superior to concentration and microscopy for hookworm spp. and *Strongyloides* spp. [12, 55]. However, comparisons between charcoal culture and other specialist diagnostic techniques such as the Baermann-Moraes method and agar plate culture (APC) have been very limited and direct comparison is hampered by variations in laboratory techniques. Comparable sensitivity to APC, and superior sensitivity to Baermann have been reported [56], as has inferior sensitivity to APC [57].

It is unknown whether positive results detected by qPCR alone with negative microscopy or charcoal culture represent false positives, or true infections that would otherwise not be diagnosed. A clear example of this in the context of this study is whether the *Entamoeba cysts* seen in the samples were *E. histolytica* or more common non-pathogenic species. An additional test would be required to positively identify other *Entamoeba* species in order to establish that negative qPCR results represent true negatives. Future development of this assay would need to include the addition of other *Entamoeba* primers and probe panels.

We acknowledge that our qPCR panel does not include some important pathogens previously detected via microscopy in the Nepalese migrant population, for example *Hymenolepis nana* [12] . The selection of targets for a multiplex PCR often means that some parasites are not included, and traditional diagnostic methods remain an important way of diagnosing these infections [58]. We also note that *A. lumbricoides* was detected on only a single occasion, and that assessing a precise sensitivity of our qPCR for this pathogen would require a larger sample size.

In this paper, we suggest that combining molecular and traditional techniques can reduce the number of stools needed to achieve acceptable sensitivity, and that both methodologies have important and complementary roles. There is concern that the increasing availability of molecular methods will lead to loss of expert microscopy skills [59].

We found that for several GIP (hookworm spp., *Strongyloides* spp. and *G. duodenalis*) traditional methods detected cases not found by qPCR alone, and our data support the assertion that concentration and helminth culture methods still have a vital part to play in GIP diagnosis.

While intensity of infection was not quantified, it was generally noted to be low. In this scenario, trophozoites of infective protozoa are less likely to be detected via microscopy, and cyst, ova, and larvae numbers may be lower. DNA extraction utilises only a small volume of stool, and in low burden infections this may contain a low quantity of genetic material, leading to false negatives. For *S. stercoralis* in particular, PCR has been suggested to have poor sensitivity compared with traditional diagnostic methods [(60)], with many assays not extensively validated, although the assay we used is the one of the most widely used and studied [(26, 29)].

It is also important to consider the nature of the participants on interpretation of results. In our study, participants were healthy and asymptomatic, having recently undergone an arduous military selection process. The results are most applicable therefore to our setting, namely screening in asymptomatic migrants or returning travellers from an endemic setting, and sensitivity and specificity may differ in those who are symptomatic.

Finally, a further limitation of the study is that it did not cover the economic differences between the proposed reference standard of three stools against single faecal analysis with the addition of multiplex qPCR. Whilst the simplification of logistics and reduction of time spent screening samples are clear when comparing three versus a single stool, any cost benefit in using the novel method has not been established. Comparative cost effectiveness will be explored following further evaluation.

Conclusions

Our findings demonstrate that addition of qPCR enhances the diagnosis of *Giardia duodenalis* and helminth infections such as *Strongyloides* spp. and hookworm spp. when analysing a single stool sample. This substantially improved the diagnostic yield in a setting of chronic, asymptomatic infections, where traditional diagnostic methods have poor sensitivity. The combined hybrid approach needs further evaluation for the investigation of symptomatic infections and in settings where other gastrointestinal parasites are more prominent. The ability to obtain reliable results from a single stool sample has potential applications both for remote, austere environments where repeat sampling may be impossible, and for busy laboratories where specialist parasitological methods may not be

available. It also has the advantage of removing the need for repeated cultures, reducing the requirement for laboratory staff to work with potentially infectious invasive L3 larvae. Obtaining reliable results from a single stool sample reduces patient discomfort, laboratory resources consumed, and potentially cost. However, at present, equipment availability limits qPCR use beyond well-resourced, high-income projects and laboratories.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Highlights

- Accurate diagnosis of GIP by traditional methods requires multiple faecal samples
- Additional of faecal multiplex qPCR to traditional methods improves GIP detection rates
- Faecal multiplex qPCR identifies more GIP infections than traditional methods
- A hybrid molecular/traditional approach on one stool sample gives comparable sensitivity to three traditionally tested samples