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Original Article

Agar Plate Culture: An Alternative Sensitive Routine Laboratory Detection Method for *Strongyloides stercoralis* and Hookworm Parasites

*Tadesse HAILU¹, Endalkachew NIBRET², Arancha AMOR³, Abaineh MUNSHEA²,
Melaku ANEGAGRIE³

1. Department of Medical Laboratory Science, College of Medicine and Health Sciences, Bahir Dar University, Bahir Dar, Ethiopia
2. Biology Department, Science College, Bahir Dar University, Bahir Dar, Ethiopia
3. Mundo Sano Foundations, Institute of Health Carlos III, Madrid, Spain

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*Correspondence Email:
tadessehailu2005@yahoo.com

Abstract

Background: Human infection with *Strongyloides stercoralis* and hookworm parasites is usually under reported due to less sensitive diagnostic methods. Agar plate culture (APC) is the most sensitive technique for parasites having larval stage. However, using APC in routine diagnosis is uncommon. This study aimed to determine the detection rate and sensitivity of APC in comparison with formal ether concentration technique (FECT) and spontaneous tube sedimentation techniques (STSTs) for *S. stercoralis* and hookworm larvae.

Methods: Stool samples collected from 844 schoolchildren in Amhara Regional State, northwestern Ethiopia in 2019, transported to nearby health institutions and processed by APC, FECT and STSTs. The prevalence of *S. stercoralis* and hookworm was computed by descriptive statistics and Chi-square. The diagnostic agreement among the three techniques was evaluated using Kappa value.

Results: The overall prevalence of *S. stercoralis* and hookworm infections by combining the three methods was 13.2% (111/844) and 33.8% (277/844), respectively. Using APC alone, the prevalence of *S. stercoralis* and hookworm were found to be 10.9% (92/844) and 24.5% (207/844), respectively. Agar plate culture was 5.4 and 2.7 times respectively more sensitive than FECT and STST, with slight and fair agreement in the detection of *S. stercoralis*. Hookworm diagnostic agreement was moderate between APC and FECT, and APC and STST. The Kappa value between STST and FECT diagnostic methods was substantial.

Conclusion: APC has a better detection rate of *S. stercoralis* and hookworm larvae. Therefore, APC can be used as an alternative routine diagnostic method to *S. stercoralis* and hookworm co-endemic countries.



Introduction

Strongyloides stercoralis and hookworm are intestinal parasites that belong to the soil-transmitted helminths (STHs). They are found in soil polluted with feces in the wet and warm climates especially in rural communities where poor sanitation and hygiene are being practiced (1). Globally, an estimated 370 and 500 million people are affected by *S. stercoralis* and hookworm, respectively (2,3). To date, the exact burden of *S. stercoralis* is not yet known. However, in the case of hookworm infection disability-adjusted life year (DALYs) has been estimated to vary between 60,000 and 22.1 million DALYs (4).

Strongyloides stercoralis and hookworm are highly neglected intestinal nematodes (5). Human infections occur when larvae living in faecally contaminated soil penetrate the intact skin. The disease outcomes of these parasite infections are manifested by gastrointestinal complications, which lead to malnutrition, unhealthy condition, and low productivity of infected individuals (6).

Among the diagnostic methods of *S. stercoralis* and hookworm species, FECT is a simple rapid diagnostic test for the detection of *S. stercoralis* and hookworm from stool samples. However, it is less sensitive to detect the diagnostic stages of these two parasites (7) as compared to APC. The STST has better sensitivity in the detection of *S. stercoralis* and hookworm larval infections, but it is not yet adopted as a routine diagnostic technique (8). Although APC is also highly sensitive in the detection of *S. stercoralis* and hookworm larvae (9), it has not been used as a routine diagnostic method in poor countries like Ethiopia where *S. stercoralis* and hookworm are highly prevalent (10,11). Currently, a novel approach for immunodiagnosis of *S. stercoralis* infections using rSsFAR with reliable sensitivity and specificity was developed (12).

The above facts indicated a need for having improved diagnostic parasitological techniques

with high degree of sensitivity and specificity in geographical locations where *S. stercoralis* and hookworm infections are co-endemic and where limited access to more sophisticated techniques.

Co-endemic areas of hookworm and *S. stercoralis* parasites should be known using highly sensitive diagnostic techniques. Moreover, *S. stercoralis* should be included in the current STHs prevention and control program. This of course ultimately helps to minimize the burden of *S. stercoralis* and hookworm infections among human populations in co-endemic areas.

Therefore, we aimed to compare and evaluate the detection rate and sensitivity of APC, FECT and STST in co-endemic areas of the two parasites in Amhara Regional State.

Materials and Methods

Study design, area and period

A cross-sectional study was conducted among schoolchildren in Amhara Regional State, northwestern Ethiopia in 2019. Primary schoolchildren aged from 6-14 yr, volunteer to give stool and consent were included in the study. Children who had taken anthelmintic drugs for the last three months before and during the data collection time were excluded.

The sample size was calculated using simple population proportion formula by taking $P=50\%$, 95% confidence interval (CI), 5% ($d=0.05$) margin of error, and 2 design effects (13).

$$n = \frac{Z^2 \cdot P(1-P)}{d^2} = 384$$

By adding 10% for none response ($384 \times 1/10 = 38 + 384 = 422$), and 2 designs effect ($2 \times 422 = 844$). Overall, 844 schoolchildren were randomly selected in each class and screened for *S. stercoralis* and hookworm infections using APC, FECT and STSTs.

Laboratory Data Collection

Approximately, seven-gram of fresh stool sample was collected from each study participant using a stool cup and was transported to the nearby health institution to confirm *S. stercoralis* and hookworm infection using FECT, STST, and APC tests. Approximately, half a gram, three-gram and three-gram of fresh stool samples were used in FECT, STST, and APC techniques, respectively. Detection and identification of the larval stages of *S. stercoralis* and hookworm species were done by light microscope. In *S. stercoralis* larvae, the buccal cavity is short and the tail is short and notched. However, in hookworm species, the buccal cavity is long and the tail region is long and pointed (14). Those schoolchildren who were found to be positive for either *S. stercoralis* or hookworm using any one of the above diagnostic tests were considered as positive.

In the FECT, about half a gram stool was processed based on a modified Ritchie's method (15) and two and half milliliters of 10% formalin and one milliliter of ethyl acetate was added in the collection tube, mixed with approximately half a gram of stool and let stood for a minute. The cover of the sample collection tube was discarded and the filtration concentration unit with conical tube was introduced. Both pieces of the device were screwed until well closed. The sample was mixed up carefully, turned over and centrifuged at 1000-rpm, for three minutes. The supernatant was removed and a small amount of the sediment was put on a glass slide, well mixed, covered with cover slide and looked for the presence of ova of hookworm and rhabditiform larvae of *S. stercoralis* using a microscope first with 10x and them with 40x (15).

In the STST, approximately three-gram of unrefrigerated stool sample was weighed and homogenized in 10 ml of normal saline solution (0.85%). The mixture was filtered through surgical gauze into a 50 ml plastic tube and then the tube was filled with more normal saline solution, plugged, and shaken vigorously. The tube was left to stand for 45 minutes. The

supernatant was discarded and a sample was taken from the bottom and put on a microscope slide. The slide was observed using a microscope to check the presence of ova of hookworm and rhabditiform larvae of *S. stercoralis* (8).

In the APC, about three-gram of stool was placed on the center of a nutrient agar-plate in a 100 × 15 mm petri dish. The petri dish was sealed with adhesive tape and incubated at 26 °C for 48 hours. The surface of the agar-plate was analyzed daily with dissection microscope or visually with naked eye for the presence of furrows/tracks of moving larvae (16). The adhesive tape was removed and then after five milliliters of a 10% formalin solution was added to the agar surface. Five minutes later, the formalin suspension was transferred from the agar plate to a conical test tube and centrifuged at 1500 rpm for 5 minutes. Finally, the sediment was looked on a microscope with 10x and then with 40x for classification of the *S. stercoralis* and hookworm larvae by identifying the buccal cavity and the tail region (17).

Comparison of the detection rate of the diagnostic methods

The detection rate of FECT, STST and APC for *S. stercoralis* and hookworm was checked. The diagnostic agreements of methods were evaluated by Kappa value, number of observed agreement, number of agreements expected by chance and standard error of Kappa. Kappa results were interpreted as follows: values ≤ 0 as no agreement; 0.01–0.20 as none to slight; 0.21–0.40 as fair; 0.41–0.60 as moderate; 0.61–0.80 as substantial and 0.81–1.00 as almost perfect agreement (18).

Data quality assurance

Training of the laboratory staffs on sample collection and diagnosis as well as an explanation about the study were given before stool sample collection. Proper labeling of the stool cup with serial numbers was done. The amount of stool sample was checked during collection and transported immediately to the

nearby health institution laboratory. Each test was performed by following standard operating procedure (SOP). To eliminate observer bias, smeared stool slides were examined independently by two laboratory technologists and the results of their observations were recorded for later comparison on separate sheets. The discordant results were re-checked by the principal investigator. Generally, the quality assurance was checked during pre-analytical, analytical and post-analytical stages.

Data Analyses

Data were analyzed using SPSS (ver. 23, Chicago, IL, USA) statistical software. The overall prevalence of *S. stercoralis* and hookworm infections were calculated using descriptive statistics and Chi-square at 95% CI. Kappa value, number of observed agreement, number of agreements expected by chance and standard error of Kappa were also computed at 95% CI.

Ethical clearance was obtained from the Ethical Review Committee of Science College, Bahir Dar University. Permission letters were

also secured from the Amhara Regional Health Bureau, Amhara Regional Education Bureau, Zonal and Woreda Education Offices. Written informed consent was obtained from the parents/guardians after explaining the purpose and objective of the study. Enrollment in the study was purely on voluntary basis and the study participants' laboratory results were kept confidential. Study participants who were positive for any intestinal parasite were referred to medical doctors for treatment.

Results

Socio-demographic characteristics of study participants

Overall, 844 primary school children with a mean age of 10.3 yr (range: 6–14 yr) and a standard deviation of 1.77, were included. The majority of the study participants (43.1%) were in the age category of 10-11 years. Most of the participants (88.3%) were rural dwellers and male students accounted for 51.7% (Table 1).

Table 1: *Strongyloides stercoralis* and hookworm infections across socio-demographic variables of primary schools students using combined diagnosis results of FECT, STST, and APC

Variables		Total examined N (%)	<i>S. stercoralis</i>		Hookworm spp.	
			Positive (N,%)	Negative (N,%)	Positive (N,%)	Negative (N,%)
Age (yr)	6-9	254 (30.1)	23 (9.1)	231 (90.9)	74 (29.1)	180 (70.9)
	10-11	364 (43.1)	53 (14.6)	311 (85.4)	118 (32.4)	246 (67.6)
	12-14	226 (26.8)	35 (15.5)	191 (84.5)	88 (38.9)	138 (61.1)
Sex	M	436 (51.7)	63 (14.4)	373 (85.6)	154 (35.7)	282 (64.3)
	F	408 (48.3)	48 (11.8)	360 (88.2)	123 (30.1)	285 (69.9)
Residence	Urban	99 (11.7)	12 (12.1)	87 (87.9)	32 (32.3)	67 (67.7)
	Rural	745 (88.3)	99 (13.3)	646 (86.7)	245 (32.9)	500 (67.1)
Total		844 (100)	111 (13.2)	733 (86.8)	277 (32.8)	567 (67.2)

*M=male; F= female

Detection and identification of *S. stercoralis* and hookworm in agar plate culture media

Furrows left on agar plates by larvae and free-living adults of *S. stercoralis* and larvae of hookworm were seen using dissection microscope or naked eye (Fig. 1). The tracks left by

S. stercoralis larvae were characteristically much thinner (Fig. 1A) than those left by hookworm

larvae on the agar plate culture (Fig. 1B).

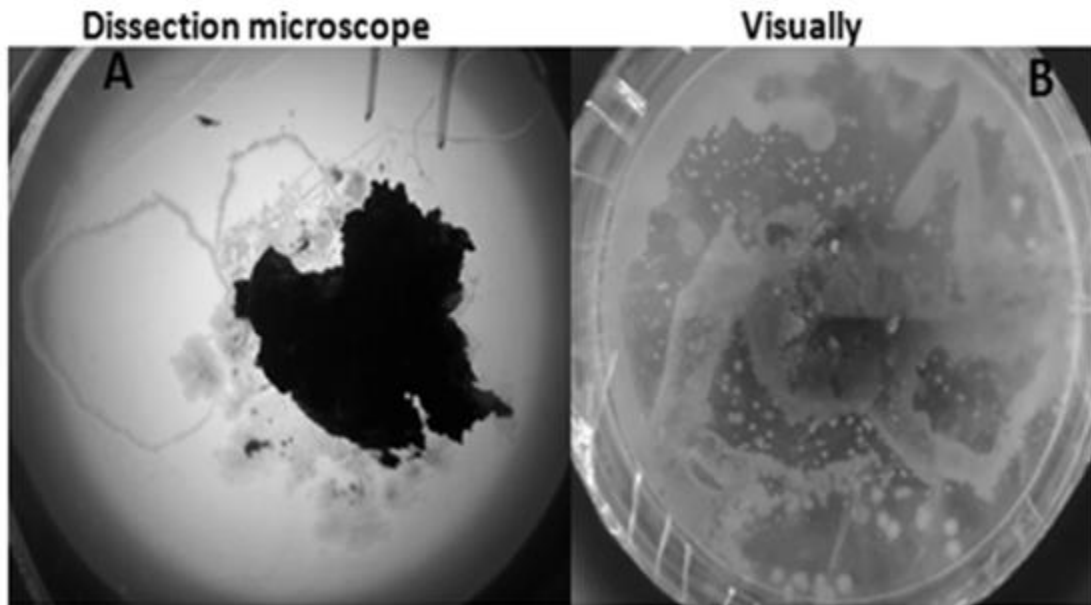


Fig. 1: The growth and crawling of *S. stercoralis* and hookworm on agar plate culture media; A= furrow left by *S. stercoralis* larvae, B= Furrow left by hookworm larvae

Identification of *S. stercoralis* and hookworm larvae was done through microscope by considering the buccal cavity and tail regions of the larvae. The buccal cavity is short in *S. ster-*

coralis larva whereas in the hookworm larva, it is long. The tail region of *S. stercoralis* is short and notched whereas the tail of hookworm larva is long and pointed (Fig. 2).

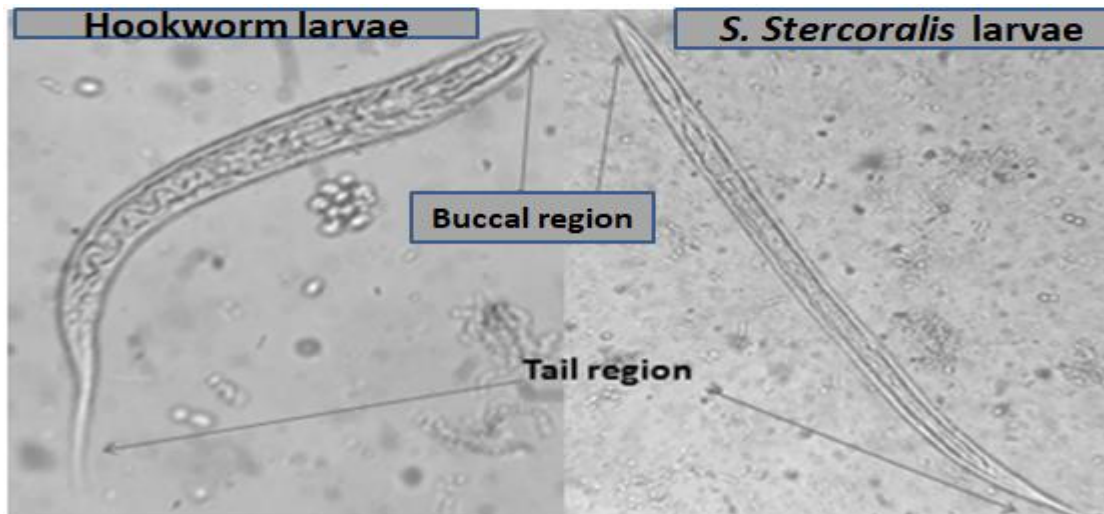


Fig. 2: The difference between *S. stercoralis* and hookworm larva

Prevalence of *S. stercoralis* and hookworm

The overall prevalence rates of *S. stercoralis* and hookworm infections using a combination of FECT, STST and APC methods were 13.2% (95 % CI:11.0-15.6%) and 32.8% (95 % CI: 29.74-36.06%), respectively (Table 1). The prevalence of co-infection of *S. stercoralis* and hookworm was 5.1% (43/844). High prevalence rates of *S. stercoralis* (10.9%) and hookworm (24.5%) were recorded by APC. Agar plate culture was respectively 5.4 and 2.7 times more sensitive than FECT and STST in the

detection of *S. stercoralis*. However, APC was almost equally sensitive as the other two techniques in the detection of hookworms. Similarly, STST was almost equally sensitive to FECT in the detection of both *S. stercoralis* and hookworms (Table 2). Among the combination of two methods employed for diagnosis, a combination of STST and APC had the highest detection rates for both *S. stercoralis* (12.7%) and hookworm (32.5%) parasites (Table 2).

Table 2: The prevalence rates of *S. stercoralis* and hookworm parasites as diagnosed using FECT, STST, APC techniques individually and their combinations

Types of methods	Total examined (N)	<i>S. stercoralis</i>		Hookworm spp.	
		Pos (N)	% at 95%CI	Pos (N)	% at 95%CI
FECT	844	17	2.0 (1.26-3.20)	181	21.5 (18.81-24.35)
STST	844	34	4.0 (2.90-5.58)	199	23.6 (20.84-26.56)
APC	844	92	10.9 (8.92-13.18)	207	24.5 (21.75-27.54)
FECT+STST	844	42	5.0 (3.71-6.66)	246	29.2 (26.18-32.30)
FECT+APC	844	98	11.6 (9.62-13.95)	255	30.2 (27.21-33.39)
STST+APC	844	107	12.7 (10.60-15.10)	274	32.5 (29.39-35.69)
FECT+STST+APC	844	111	13.2 (11.04-15.60)	277	32.8 (29.74-36.06)

*Pos = Positive

Diagnostic agreement of methods

The diagnostic agreement between APC and FECT (kappa value=0.174), and APC and STST (kappa value=0.258) to detect *S. stercoralis* was slight and fair, respectively. The agreement between STST and FECT in the diagnosis of *S. stercoralis* was fair (Kappa

value=0.335). The number of observed agreements between APC and FECT and that of APC and STST was comparable in the detection of *S. stercoralis*. However, the agreement was high between STST and FECT (Table 3).

Table 3: The diagnostic agreement of FECT, STST, and APC techniques in *S. stercoralis* detection

Methods	APC		Kappa value (95%CI)	NOA (N,%)	NAEC (N, %)	SEK	χ^2 , P-value
	Pos	Neg					
FECT	Pos	11	0.174 (0.077-0.270)	757 (89.69)	738.7 (87.52)	0.049	51.7, 0.000
	Neg	81					
STST	Pos	19	0.258 (0.153-0.363)	756 (89.57)	725.4 (85.95)	0.054	73.8, 0.000
	Neg	73					
FECT	STST		0.335 (0.165-0.505)	811 (96.09)	794.4 (94.12)	0.087	107.4; 0.000
	Pos	9					
	Neg	25	802				

**NOA=Number of observed agreement, NAEC=Number of agreements expected by chance, SEK=Standard error of Kappa, Pos=Positive, Neg=Negative

The diagnostic agreement between APC and FECT (kappa value=0.592), and that of APC and STST (kappa value=0.540) to detect hookworm was moderate. The Kappa value between STST and FECT in the diagnosis of

hookworm was substantial (kappa value=0.620). The number of observed agreements between APC and FECT, APC and that of STST and STST and FECT to detect hookworm was comparable (Table 4).

Table 4: The diagnostic agreement of FECT, STST, and APC techniques in hookworm detection

Methods		APC		Kappa value (95%CI)	NOA (N, %)	NAEC (N, %)	SEK	χ^2 , P-value
		Pos	Neg					
FECT	Pos	133	48	0.592 (0.527-0.657)	722 (85.55)	544.8 (64.55)	0.033	298.3, 0.000
	Neg	74	589					
STST	Pos	132	67	0.540 (0.473-0.606)	702 (83.18)	535.6 (63.47)	0.034	245.9, 0.000
	Neg	75	570					
FECT	Pos	134	47	0.620 (0.556-0.684)	732 (86.73)	549.4 (65.09)	0.033	325.5, 0.000
	Neg	65	598					

*NOA=Number of observed agreement, NAEC=Number of agreements expected by chance, SEK=Standard error of Kappa, Pos=Positive, Neg=Negative

Discussion

Strongyloides stercoralis and hookworm are public health important helminths, which are co-endemic in tropics and sub-tropics including Ethiopia (11). The prevalence of *S. stercoralis* in the current study was 13.2%. This result is in agreement with 12.7% in south-central Côte d'Ivoire (19), but it is lower than 48.6% reported in Cambodia (20). This difference could be due to the difference in the sanitation and shoes wearing habit, working with bare hands, and the variation in the combination of methods used for the diagnosis of *S. stercoralis*.

On the other hand, in the present study, the prevalence of hookworm (34.9%) is lower than 41.3% in northwest Ethiopia (21), 51.0% in south-central Côte d'Ivoire (19) and 49.0% in Cambodia (20). The low prevalence in the present study might be due to differences in sanitation, shoes wearing habit, working with bare hands local endemicity of the parasite and employment of different laboratory diagnosis.

The detection rate of FECT to *S. stercoralis* in the current study was low 2.0%. This result is comparable with previous findings from rural

Bahir Dar, Ethiopia (3.5%) (11), but it is lower than previously reported (12.3%) in Addis Ababa, Ethiopia (22). The low detection rate of *S. stercoralis* by FECT might be attributed to several factors. For instance, the amount of stool sample used in the procedure, the sampling technique, filtration of the stool suspension through the gauze that might remove the larvae in the final sediment and also the immune status of the study participants.

In the present study, APC was superior to the FECT in the detection of *S. stercoralis*. This current result was consistent with previously conducted researches (9, 22-24). This high detection rate by APC might be ascribed to the use of large amount of stool sample (three-gram), the longer 48 h incubation time which might have helped the *S. stercoralis* larvae to emerge from stool, as well as the absence of debris coming from fungus growing on the APC medium.

Hookworm can be diagnosed by different parasitological diagnostic methods with different sensitivity. In the present study, APC showed the highest detection rate for hookworm larvae. This result is consistent with former study conducted in East Sikkim, India (25). Similarly, in the present study, APC had

better sensitivity than FECT in detection of hookworm larvae. This finding is consistent with previous reports (26,27).

In the present study, the detection of hookworm by STST was comparable with APC in a 1:1 ratio, but it was superior in the detection of *S. stercoralis* (2:1) ratio compared with FECT. This high detection rate of *S. stercoralis* and hookworm by STST in the present study is supported by the previous study conducted in Peru (8). Although STST has better performance in the detection of *S. stercoralis*, application of this test in routine diagnostic activity is limited.

Conclusion

From the three techniques, APC is superior to the other two techniques in detection of *S. stercoralis* and hookworms. The STST is also better in sensitivity than FECT. Therefore, APC can be recommended for epidemiological and routine clinical diagnosis in places where *S. stercoralis* and hookworm are endemic.

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Conflicts of interest

Authors declare that they have no competing interests.

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