International Journal for Multidisciplinary Research (IJFMR)

E-ISSN: 2582-2160 • Website: www.ijfmr.com • Email: editor@ijfmr.com

First Report on Accurate Diagnostic of Hookworms and *Strongyloides stercoralis* Based on Different Diagnostic Approaches in Patients with Gastro-intestinal Disorders in Northeastern Himalayan State of India.

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Abstract

Background & objectives: Hookworm infection is one of the important causes of iron deficiency anaemia in children. Sensitive diagnostic methods are important for accurate assessment among the different species of hookworm infection. Microscopy is still gold standared however microscopy alone cannot differentiate between hookworm species and other similar Strongyloid. Objective: The objective of this study is to identify the hookworm eggs from the stool sample by microscopy and further subjected for species specific identification targeting mitochondrial cytochrome oxidase I gene. Methods: A total methods of 300 samples were collected from the children with gastrointestinal symptoms during February 2018 to February 2019.All samples were examined using saline and iodine mount preparation and further subjected to different culture techniques and conventional PCR.Stoll dilution eggs counts technique was used to identify the intensity of the hookworm positive samples Results: Stool samples with intestinal parasites detected in this study were 70/300;out of which 20 samples was hookworm positive by microscopy. Sixteen out of 20 were identified as Necator americanus and 2 samples each were identified as Anylostoma duodenale and Strongyloides stercoralis. There were no mixed infections in this study. Stoll's dilution egg count showed moderate infection in 66.6%, light and heavy infection in 16.7%. Diarrhea accompanied with dehydration, weakness, fever, bloating was the most common complained associated (6.6%) among the hookworm positive children. Interpretation & Conclusion: The highest infection was seen in 6-10 years and more common in males (p=0.7648).Species identification of hookworm targeting COX I gene is rapid and sensitive for accurate identification for the hookworm species in the limited resource setting area like Sikkim.

Introduction:

Hookworm infection is spread globally and causes by two species namely, *Ancylostoma duodenale* and *Necator americanus* [1]. *A.duodenale* is common in Middle East, North Africa, India, Australia and Europe and *N.americanus* is more common in the South East Asia [2].

In India, *N.americanus* is seen in Southern parts of India & *A.duodenale* in Northern India [3]. In 2010, the estimated global burden of intestinal helminthiasis was 5.2 million (Mio) disability adjusted life years (DALY) lost in which hookworm diseases accounts for the biggest part of the burden estimates (3.2 Mio DALYs) [4,5,6].

Even though they are grouped together as hookworm in control strategies in which the drugs of choices are similar, it is important to know their life histories which are important epidemiologically[7]. The species *A.duodenale* can causes a much greater degree of intestinal blood loss, more virulent and causing anaemia [2]. (De Silva NR, 2003).

It is noted that a single *A.duodenale* worm ingests about 150µl (0.15ml) of blood per day and *N.americanus* worm about 30µl (0.03ml) [8]. Depending on the status of host iron, intensity of the infection of 40 to 160 worms per individuals is associated with hemoglobin level below 11g per deciliters [9].

Currently different diagnostic methods are available; however there are number of drawbacks and technical limitations. Direct smear examination of stool alone cannot differentiate between the hookworms' species and the other similar species like Strongyloid nematodes.

The Kato-Katz technique is comparatively cheap and simple method but lacks sensitivity for the detection of low-intensity soil transmitted helminthes infections [10, 11]. There are various coproculture techniques that are employed to obtain the filariform larvae to differentiate between the *A.duodenale*, *N.americanus* and *Strongyloides.* These methods are easy and less expensive, although time consuming and need well experience technician to expertise it [9, 12, 13, and 14]. FLOTAC technique for the detection of helminthes eggs has higher sensitivity however it requires more sophisticated laboratory equipments and is a relatively low throughput method [15, 16, 17, 18, 19].

Different molecules technique has provided accurate tools for the species identification of hookworm. PCR targeting internal transcribed spacer (ITS) – 1 and -2 regions and the 5.8s region, also mitochondrial cytochrome oxidase subunit I gene (COX-1) sequences have used to detect and identification of hookworm from the human and animal feces [20,9,21,22,23].

However in terms of cost effective and easier it can be used to design species-specific primer sets which amplify PCR products at the COX I gene, which easier to isolates the species[24].

Therefore, this study aims to identify the species of hookworm based on the COX 1 gene which is species -specific direct PCR method to differentiate between *N.americanus* and *A.duodenale*, and also to rule out the *Strongyloides* species, 18S rRNA gene were targeted as the secondary objective. This study will able to determine the problem of hookworm, its species identification based on the molecular method in this part of the India which are still lacking. In addition, the intensity of worm burden and blood parameters which includes estimation of Haemoglobin, Pack Cell Volume (PCV), Mean Corpuscular Haemoglobin Concentration (MCHC), Mean Corpuscular Haemoglobin (MCH) and eosinophil count were also analyzed in hookworm positive patients.

Material and methods:

Study area and population

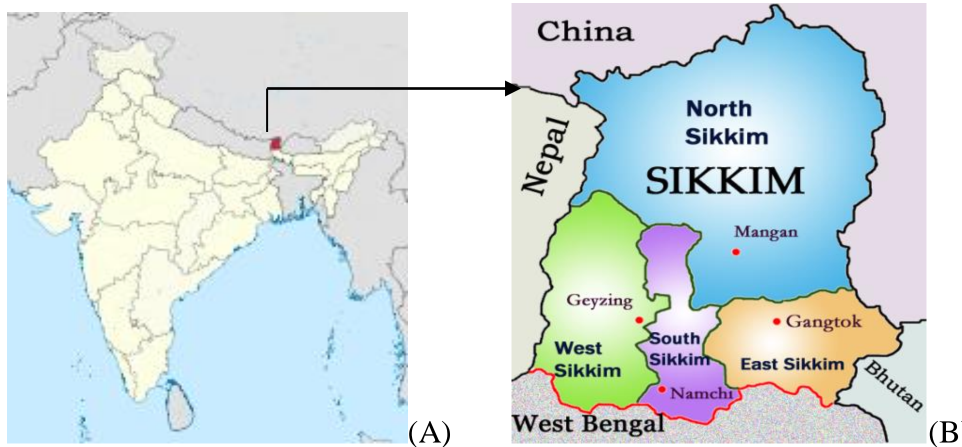

Figure 1: Location of study areas in North eastern state of India. (TUBS [CC BY-SA 3.0 (https://creativecommons.org/licenses/by-sa/3.0) and map of Sikkim (https://images.app.goo.gl/NsBkXzajS6wtG9r26)

Materials and methods

Study type and period

A prospective study was carried out in Sikkim, India , in children in 1-15 years(Fig 1).

A total of 300 stool samples were collected from children attending CRH and STNM hospitals with complaints of gastrointestinal symptoms, collected before radiological examinations using barium or anti-diarrheal medications that could interfere with parasite identification. This study was approved by University ethical committee registration number IEC/309/15-032.

Sample collection and laboratory procedures

The children were given a labeled, leak proof container with a plastic scoop (Hi media) to collect sample as per standards procedure of the WHO and processed within 2 hours of collection. Then the samples were examined by direct smear examination. Stool samples after microscopy were further subjected to Harada Mori culture (HMC), agar plate culture method (APC) and modified agar plate culture (HAPC) for their larval nematodes (L3) and PCR targeting COX I gene for hookworm's species specific identifications. Stoll's dilution egg counting technique was also performed to identify the intensity of hookworm infection among the children. Second samples were collected for backup in case of contaminated during culture. Blood parameters were done using $A^{C}T^{TM}$ 5 diff Cap Pierce Hematology Analyzer.

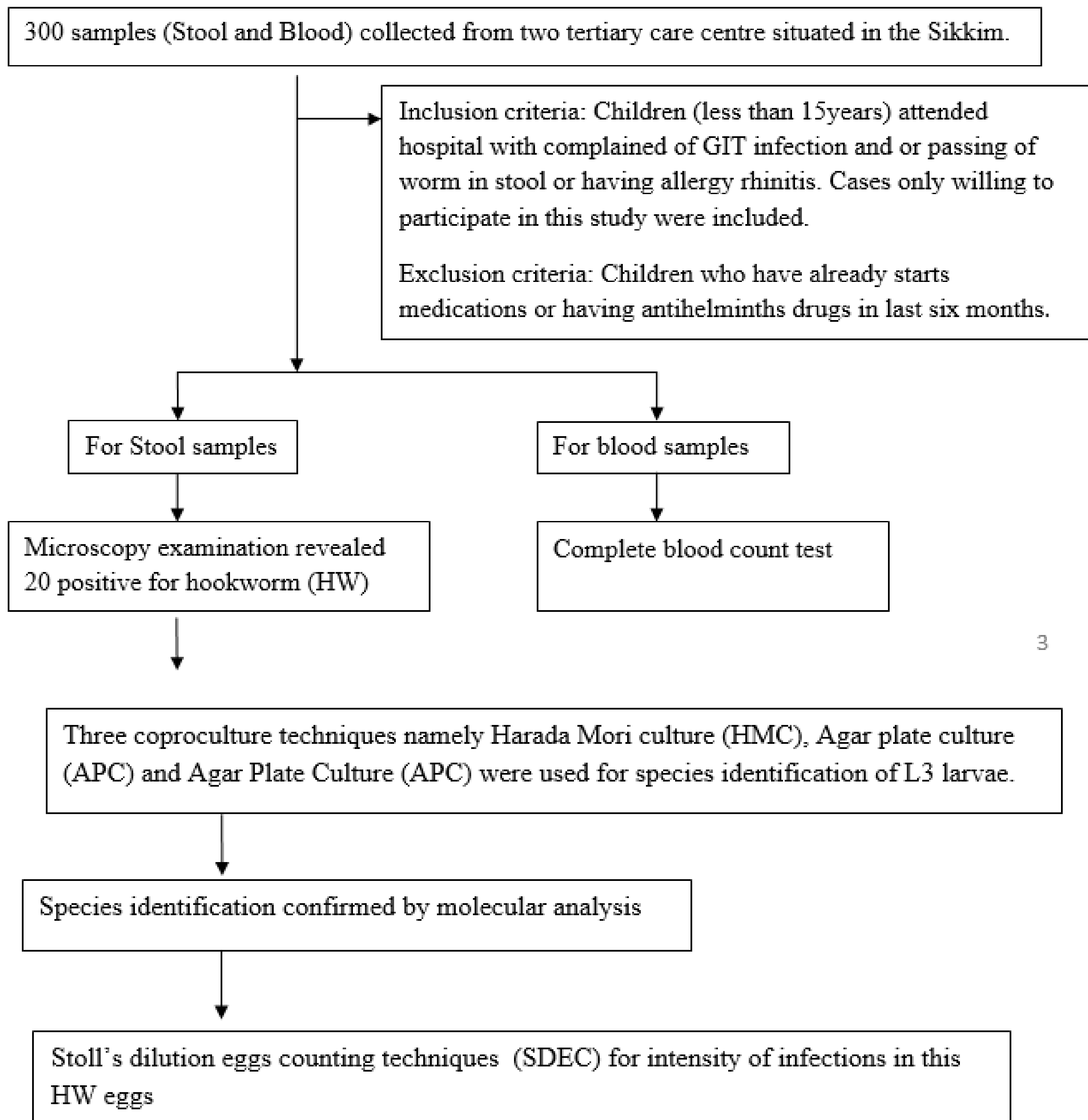

Fig: 2. Study design.

Study design.

Informed written consent from parents/guardians was procured on the proforma . Total 300 stool and blood samples were collected from children of age group to 15 years. Of the 300 stool samples, 20 stool samples were positive for HW eggs for further analysis and blood samples for CBC analysis.

Stool culture

About 1gram of fresh stool samples was cultured to the rabditiform larva at 25 to 28°C by the HMC technique [25]. The tube was kept for 7 to 10 days and checked daily. For APC, approximately 2 gram of samples were used and incubated at 26 to 33°C for 2 days [26]. In a case of MAPC, a canal of 1cm wide is made and 2gram (approximately) of samples were smeared on the agar and incubated at 26 to

33°C for 2 to 3 days as described by Khanna V *et al.,*2015[27]. The observation was made in concordance with the remarks made by Santos N *et al*, 1993 and Jongwutiwes *et al.,* 1993. [28, 29]
Helminthes eggs count by SDEC technique.
Quantitative hookworm egg counts were obtained by SDEC (Garcia LS, 2011) and expressed as eggs per grams (epg). The helminthic epg were assayed according to Knopp S *et al*., 2008. The egg count was multiplied by 100 to obtain the number of eggs per gram of stool.. Samples which were contaminated with maggots after inoculation of 2nd or 3th day were again reinoculated with the second fresh samples.

DNA extractions:
DNA was extracted directly from the specimen using commercial kit Helini pure fast stool DNA Minispin prep kit (Catalog no.2006; *Helini biomolecules*, Chennai, India) following the manufacturer's instructions..

PCR amplification:
Species specific conventional PCR method was performed to amplify the mitochondrial cytochrome COX I gene in human hookworm. The forward and reverse primers used for *N.americanus* were NaF: (5′-TTC GTT TGG AGT TGG CT) and NaR: (5′-TAG CTC CAG CCA AAA CT) and for *A.duodenale* AdF: (5′-TTC GTT TGA GTT GGC A) and AdR: (5′-TGG CAC CAG CCA ATA CA) to amplify 550bp (*N.americanus*) and 300bp (*A.duodenale*) as previously described [7]. The forward and reverse primers used for *Strongyloides* were Strol 18S-1530F: (5′-GAA TTC CAA GTA AAC GTA AGT CAT TAG C) and18S-1630R: (5′-TGC CTC TGG ATA TTG CTC AGT TC) to amplify 180bp [30]. PCR reaction mixtures consisted of 10µl of Red Dye PCR Master Mix (*Helini* biomolecules), 5µl of each primer pairs and 5µl of extracted DNA. The reactions were carried out in 20µl volume. The amplification condition was as follows, the first initial denaturation at 95ºc for 5 minutes, followed by 35 cycles (denaturation at 95ºc for 30 seconds, annealing at 58ºc for 30 seconds, extension for 72ºc for 30 seconds and final extension at 72ºc for 5 minutes. All PCR reactions were carried out using Eppendorf Master Cycler.
10µl of sterile water was used as a negative control and 10µl of positive template (*Helini* biomolecules). PCR products were electrophoresed on 2% agarose gel containing ethidium bromide and visualized under UV transilluminator.

Specificity
The primers have been designed for the specific and exclusive in vitro detection of *N. americanus*, *A.duodenale* and *Strongyloides* species. The target sequence (*N.americanus* [COX 1 gene]. *A.duodenale* [COX 1 gene] & *Strongyloides* species [18S r RNA gene] is highly conserved used for designing. The primers and probe in this kid have 100% homology with a broad range of clinically relevant reference sequences based on a comprehensive bioinformatics analysis.

Statistical analysis:
GraphPad software (San Diego, USA) was used to analyze categorical variables with Chi-square or Fisher's exact tests as appropriate. Different variables were summarized using frequency tables. A p-value ≤ 0.05 was considered statistically significant.

Sensitivity and specificity of different coproculture techniques were calculated using MedCalc Software.

Results:

Study population characteristics

A total of 300 patients were participated in this study, 190 of which were males and 110 were females. Out of this, 12 males and 8 females were positive for hookworm's eggs by microscopy. The highest hookworm infection rate was seen in 6-10 years .The infection was more common in males compare to females but statistically not significant (p= 0.7488) (Table I).

Performances of diagnostic approaches

Out of 20 samples were hookworms positive by microscopy, by culture method, 18/20 samples were identified as *Necator americanus* and 2/20 samples were *Strongyloides* (Table II). There were no *Ancylostoma spp* detected by culture. The sensitivity of HMC showed 35.0%, specificity 100%, APC showed sensitivity 85%, specificity 100% and MAPC showed 70.0% sensitivity and specificity 100% (Table: III, IV, V). L3 filariform larvae were identified during 3rd and 4th day of inoculation. In PCR method, 16/20 samples were identified as *N.americanus* and 2/20 samples were identified as *A.duodenale* and 2/20 samples were *Strongyloides*. The sensitivity of PCR showed 100%, Specificity 100%. SDEC of HW eggs showed moderate infection (Eggs=600-1000) in 51.3%, light (100-500) in 28.2% each & heavy infections in 20.5% (>1000) (Table VI).This hookworm intensity were analyzed with the WHO threshold [31].

In terms of total time taken to hatch the filariform larva, APC and MAPC takes almost similar time period of around 2 to 5 days, in some cases MAPC takes only 2 days. However, HMC takes 7 to 10 days. However conventional PCR takes 4 to 6 hours.

Blood parameters of the children with positive IPIs showed lower hemoglobin in females (46.8%), higher PCV (Hct) (34%) in females and low MCH and MCHC in males 48.9% and 57.4% respectively. However higher eosinophil count was seen more in males (42.5%) (Table VII). Diarrhea accompanied with dehydration, weakness, fever & bloating was the most symptoms at presentation (66%). However there was no correlation between the frequencies of symptoms and parasite burden.

Discussion:

In this study parasitic infection was 23.3% in which hookworm infection was 6.6%. Other predominant intestinal parasites found in this study were *A. lumbricoides* (5.3%), *G. intestinalis* (4.6%), *Entamoeba spp*. (3.3%), *Taenia spp*. (2.3%), *T.trichura* (0.6%) and *E.vermicularis* (0.3%).

Microscopy examination showed that 20/300 (6.6%) of the children were infected with hookworms in this study. These findings has been consistent with other studies done by Chidambaram C *et al*., 2017, (6.2%), Ngui R *et al*., 2012 (9.1%), Shahid SB *et al*., 2010 (10.15%), Humphries D *et al*., 2013 (39.1%) [9,20,32,33]. However these studies were based on the single fecal examination which possibly missed the chances of the actual prevalence rate. The low prevalence rate of HW in this study could be due to administration of 400mg every after 6 months in children.

Coproculture results yielded 18/20 samples as *N.americanus*, 2/20 samples as *Strongyloides* in our study. This identification of larvae isolated was in concordance with the remarks made by dos Neto, 1993, Jongwutiwes *et al*. 1999 and Chidambaram C *et al*., 2017 [28,29,9]. In the similar studies done by Shahid SB *et al.,* 2010, they found 10.15% hookworm; out of this 11.53% were *A.duodenale* and

88.47% were *N.americanus*, Parija SC *et al*., 1992 found 53.6% were *A.duodenale*, 43.7% were *N.americanus* & 2.7% were mixed infections of both [32,34]. Adenusi AA *et al*., 2003 found 6.1% were *A.duodenale*, 68.2% were *N.americanus* and 25.7% of mixed infection of both [35]. All these studies were based on Harada Mori culture method.

Compare to the HMC and MAPC, APC showed higher positive rates. In terms of sensitivity in our study, APC showed 92% compared to 69% by MAPC and 30.7% by HMC. For specificity, true negative value was 280; this calculation was in concordance with the study done by Chidambaram C *et al*., 2017[9]. Khanna *et al*., 2015, reported more parasites were found by modified method, which is contrast with our findings. Culture techniques are successful only when the larvae present are viable and chances of infections can arises in case of mishandling.

In some of the recent studies which were based on PCR method, Ngui R *et al*., 2012 found, out of 47 microscopy samples were identified 76.6% as *N.americanus* [23]. George S *et al*., 2015 found 82.9% were *N.americanus*, 17.1% were *A.duodenale* [36]. Study done by Inpankaew T *et al., 2014, found* 51% were *N.americanus* [37].

In another study done by Chidambaram C *et al*., 2017' out of 16 hookworm samples subjected for PCR, all of them were identified as *N.americanus* [9]. All these studies were based on the detection of internal transcribed spacer (ITS) -1 and – regions and the 5.8S region to detect and characterize hookworm infections directly from eggs in humans and animal feces. This study could not identify *A.ceylanicum*, the only species of zoonotic hookworm known to produce infections in humans. This could be due to the family or communities lived near household pets, it is highly chances that the *Ancylostoma* spp. identified in this study could be a mixture of *A.ceylanicum* and/or *A.duodenlae*. Beaber PC *et al*., 1984 and Hoagland KE *et al*., 1978 have mentioned that geographic variance in the distribution of the HW species depends on parasite behavior, ethnicity, climate, temperature and environmental factors [38,39].

The infection rate was more in 6 to 10 years and the maximum were males. . Generally, boys being more playful, more exposed to infection, and children playing with domestic animals, playing barefoot, having contact with fecally polluted soil, and poor personal hygiene could explain this.However in the study done in Malaysia, hookworm infection was more prevalent in females (62.7%) compare to males (37.3%) which is contact to our findings Ngui R *et al*., 2012 [29].

Majority of the children (66.6%) in this study have moderate fecal egg count. In some other studies, there has been a reported of mostly light intensity. The moderate intensity in our study could be due to family live in poor environmental sanitation and poor hygiene despite of deworming among the children and also the children are symptomatic.

Prevalence of anaemia was higher in females (46.8%) compare to males (36.1%). Preschool and school children have the greatest physiological demands for iron. Also the infection rate was more in 6 to 10 years and maximum were males. However in the study done in Malaysia, hookworm infection was more prevalent in females (62.7%) compare to males (37.3%). Higher prevalence in male in our study might be due to close with household pets, playing with bare foots, frequent contact with polluted soil and less care about the hygiene [9]. Depending on the status of host iron, a hookworm burden of 40 to 160 worms per individuals is associated with hemoglobin level below 11g per deciliters [29].

Complete blood count revealed 40% of the children from the hookworm positive were low hemoglobin level (<11g/dl) which may lead to the intensity of infections rises as the hookworm ingests blood. Among the hookworm species, *A.duodenale* causes more blood loss than *N.americanus*. However in this study lower level of Hb were observed in case of children with *N.americanus* infections. It has been

estimated that a single *A.duodenale* worm ingests about 150µl (0.15ml) of blood per day and *N.americanus* worm about 30µl (0.03ml) [36]. But MCH and MCHC decreases may be due to malnutrition of the children and there was increase in the eosinophil count which may be the reason of allergy or parasitic infection.

PCR targeting COX I gene for hookworm species identification is technically straightforward and rapid then the methods based on PCR-RFLP that require polyacrylamide gel electrophoresis and cost effective when there is no need for sequencing single specimen when investigating many isolates. In this study, *N.americanus* was predominant among the hookworm species in Sikkim; however a larger number of samples need to be studied if this observation is true. Timely diagnosis and identification of the etiological agents of gastrointestinal tract infection (GIT) in young children is important towards the benefits on de-worming, in which young children are at the higher risk of anemia and wasting malnutrition, which may be most vulnerable to the deleterious effects of parasites.

Community characteristics, such as water supply and soil type, type of drinking water resources may modify the effect of individual and household risk factors on the prevalence of parasitic infections. Environmental, demographic and behavioral factors will impact the effectiveness of environmental sanitation to control the spread of enteric pathogens and must be considered when planning and implementing sanitation interventions. Conclusively, the examination of personal hygiene, improving environmental sanitation, food and water hygiene, appropriate health education as well as routine medical examination and treatment is strongly recommended in children.

Table I: Study population characteristics

Gender	Total children	Positive	Negative	X^2	p-value
Male	190	12	178	0.1025	0.7488
Female	110	8	102		
Total	300	20	280		

$P \leq 0.05$ is considered significant.

Table II: Comparisons of Harada-Mori culture, Agar plate culture & Modified agar plate culture method

Characteristics	HMC	APC	Chi-square	P value
Positive	7	20	19.259	**P* value = <0.0001
Negative	13	0		
Total	20	20		
	HM	MAP	4.912	**P* value = 0.0267
Positive	7	14		
Negative	13	6		
Total	20	20		
	APC	MAP	7.059	**P* value = 0.0079
Positive	20	14		
Negative	0	6		
Total	20	20		

*Fisher exact two-tailed

Table III: Sensitivity and specificity of diagnostic test considering direct smear examination as gold standard.

Diagnostic approach	Direct smear examination		Sensitivity	Specificity
HMC	Positive	Negative	35.0%	100%
Positive	7(TP)	0(FP)		
Negative	13(FN)	280(TN)		
Total	20	280		
APC			85%	100%
Positive	17(TP)	0(FP)		
Negative	3(FN)	280(TN)		
Total	20	280		
MAPC			70%	100%
Positive	14(TP)	0(FP)		
Negative	6(FN)	280(TN)		
Total	20	280		
PCR			100%	100%
Positive	20(TP)	0(FP)		
Negative	0(FN)	80(TN)		
Total	20	280		

Table IV: Results of Stoll's dilution egg count

Number of cases with helminth infection	Helminth fecal count egg per gram(epg)		
	100-500 (light infection)	600-1000 (moderate)	>1000 (heavy)
39	11(28.2%)	20(51.3%)	8(20.5%)

Table V: Blood parameters among positive for hookworms infections.

Hb				Hct				MCHC				Eosinophil			
NV		AbV		NV		AbV		NV		AbV		NV		AbV	
>11.5g/dl		<11.5g/dl		>35%		<35%		>31.0gm/dl		<31.0g/dl		<0.04		>0.40	
Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
5 (25%)	0	3 (15%)	8 (40%)	6 (30%)	1 (5%)	2 (10%)	7 (35%)	3 (15%)	6 (30%)	5 (25%)	2 (10%)	0	4 (20%)	8 (40%)	4 (20%)

0	-	2 (10%)	-	1 (5%)	-	1 (5%)	-	2 (10%)	-	0	-	0	-	2 (10%)	-
1 (5%)	-	1 (5%)	-	2 (10%)	-	0	-	0	0	2 (10%)	-	1 (5%)	-	1 (5%)	-

References:

1. Hotez, P.J. One world health: neglected tropical diseases in a flat world. PLoS Negl Trop Dis: 2009; 405.
2. de Silva , N.R., Brooker, S., Hotez, P.J., Engels, D., Savioli, L. Soil transmitted helminthes infections, updating the global picture. Trends Parasitol; 2003; 19:547-551.
3. Sastry, A.S., Bhat, S.K., In: Sastry AS, editors. Small intestinal nematodes. Medical Parasitology: 1st ed.2011. Pondicherry, India: Jaypee Brothers Medical Publisher.
4. Murray, C.T.L., Vos, T., et al. Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990-2010: a systematic analysis for the Global Burden of Disease study. Lancet.2012; 380:2197-2223.
5. Christian, P., Khatry, S.K., West, K.P. Jr. Antenatal anthelmintic treatment, birth weight, and infant survival in rural Nepal. Lancet; 2004; 364:981-983.
6. Bethony, J., Brooker, S., Albonico, M., Geiger, S.M., Loukas, A.et al. Soil-transmitted helminth infections: ascariasis, trichuriasis and hookworm. Lancet.2006; 367:1521-1532.
7. Zhan, B, Li, T., Xiao, S., Zheng, F., Handon, J.M. Species-specific identification of human hookworms by PCR of the mitochondrial cytochrome oxidase I gene. J Parasitol. 2001; 87:1227-9.
8. Jan, M.S., Liu, D. *Necator*. Molecular detection of human parasitic pathogen. USA: CRC Press.2012
9. Chidambaram, C., Parija, S.C. et al. Evaluation of the utility of conventional polymerase chain reaction for detection and species differentiation in human hookworm infections. Trop Parasitol. 2017; 7(2), 111-116.
10. Booth, M., Vounastar, P., N'Goran, E.K. et al. The influence of sampling effort and the performance of the Kato-Katz technique in diagnosing *Schistosoma mansoni* and hookworm co-infection in rural Cote d'Ivoire, Parasitology.2003; 217:525-531.
11. Knopp, S., Mgeni, A.F., khamis, I.S. at al. Diagnosis of soil-transmitted helminthes in the era of preventive chemotherapy: effect of multiple stool sampling and use of different diagnostic techniques. PLoS Negl Trop Dis.2008; 2.e331.
12. Lwambo, N.J., Bundy, D.A., Medley, G.F. A new approach to morbidity risk assessment in hookworm endemic communities, Epidemiol Infect.1992; 108:469-81.
13. Polderman, A.M., Krepel, H.P., Baeta, S. et al. Oesophagostomiasis, a common infection of man in northern Togs and Ghana. Am J Trop Med Hyg.1991; 44:336- 244.
14. Yelifari, L., Bloch, P., Magnussen, P. et al. Distribution of human *Oesophagostomum bifurcum*, Hookworm and *Strongyloides stercoralis* infections in northern Ghana. Trans R Sor Trop Med Hyg 2005; 99:32:38.

15. Knopp, S., Rinaldi, L., Khamis, I.S. et al. A single FLOTAC is more sensitive than triplicate Kato-Katz for the diagnosis of low-intensity soil-transmitted helminthes infections. Trans R Soc Trop Med Hyg. 2009; 103:347-354.
16. Glinz, D., Silue, K.D. et al. Comparing diagnostic accuracy of Katz-Katz, Koga agar plate, ether concentration and FLOTAC for *Schistosoma mansoni* and soil transmitted helminthes. PLoS Negl Trop Dis.2010; 4: e 754.
17. Ziegelbauer, K., Steinmann, P., Zhou, H et al. Self-rated quality of life and school performance in relation to helminthes infection: case study from Yunnam, People's Republic of China. Parasit Vectors.2010; 3:61.
18. Habtamu, K., Degarege, A., Ye-Ebiyo, Y., Erko, 2011. Comparison of the Kato-Katz and FLOTAC techniques for the diagnosis of soil-transmitted helminthes infections. Parasitol. Inf.2011; 60:398:402.
19. Utzinger, J., Rinaldi, L., Lohourignon, L.K., Rohner, F. et al. Intestinal parasites in immigrants in the city of Naples (Southern Italy). Acta Trop.2011; 117:196-201.
20. Ngui, R., Lim, Y.A., Traub, R. et al. Epidemiological and genetic data supporting the transmission of *Ancylostoma ceylanicum* among human and domestic animals. PLoS Negl Dis.2012; 6: e 1522
21. Hu, M., Chilton, N.B., Zhu, X., Gasser, R.B. Single-strand conformation polymorphism-based analysis of mitochondrial cytochrome subunit I reveals significant substructuring in hookworm populations. Electrophoresis.2002; 23:27-34.
22. Hawdon, J.M., Li, T., Zhan, B., Blousin, M.S. Genetic structure of population of the human hookworm, *Necator americanus* in China. Mol Ecol.2001; 10:1433-7.
23. Ngui, R., Mahdy, M.A., Chua, K.H., Traub, R., Lim, Y.A. Genetic characterization of the partial mitochondrial cytochrome oxidase C subunit I (Cox I) gene of the zoonotic parasitic nematode, *Ancylostoma ceylanicum* from the humans, dogs and cats. Acta Trop.2013; 128:154-7.
24. Schwenkenbecher, J.M. et al. Discrimination of Culicoides midge larvae using multiplex polymerase chain reaction assays based on DNA sequences variation at the mitochondrial cytochrome C oxidase I gene. J.Med. Entomol.2009; 46, 610.
25. World Health Organisation. Guidelines for the evaluation of soil transmitted helminthiasis and schistomiasis at the community level. Geneva Schistosomiasis an Intestinal Parasite Unit. Division of Control Tropical Diseases. (WHO/CTD/ SIP/98.1)1998.
26. Garcia, L.S., 4th ed. Washington DC: ASM Press. Diagnostic medical parasitology,2001.
27. Khanna, V., T.Kriti, P.Y., Peralam, M., Chiranjay. Modified agar plate culture method for culture of *Strongyloides stercoralis*. Trop Parasitol. 2015;Jul-Dec; 5(2): 136-138.
28. Santos Neto, J.G., 1993. Movement of the rhabditiform larva of *Strongyloides stercoralis*. Lancet.1993;342:1310.
29. Jongwutives, S., Charoenkorn, M., Sitthichareonchai, P., Akaraborvorn, P., Putaporntip, C. Increased sensitivity of routine laboratory detectives of *Strongloides stercoralis* and hookworm by agar plate culture. Trans R Soc Trop Med Hyg.1999; Jul-Aug.
30. Basuni, M. et al. A pentaplex real-time polymerase chain reaction assay for detection of four species of soil transmitted helminthes. Am.J.Trop.Med.Hyg. 2011;84(2); 338-344.
31. World Health Organisation. Soil-Transmitted helminthiasis: Eliminating as Public Health Problem Soil-Transmitted Helminthiasis in Children: Progress Report 2001-2010 and Strategic Plan 2011-2020. Geneva.2012

32. Shahid, S.B., Wazib, A., Chowdhury, A. Identification of hookworm in stool by Harada Mori Culture. Bangladesh J Med Microbiol.2010; 04(02):03-14.
33. Humphries, D., Benjamin, T., Davey, D. Hookworm infection among school age children in Kintampo North Municipality, Ghana: Nutritional risk factors and response to albendazole treatment. Am. J.Trop. Med. Hyg.2013; 89(3), 540-548.
34. Parija, S.C., Malini, G., Rao, R.S. Prevalence of hookworm species in Pondicherry, India. Trop Geogr Med.1992; Oct; 44(4):378-80.
35. Adenusi, A.A., Ogunyomi, E.O.A. Relative prevalence of the human hookworm species, *Necator americanus* and *Ancylostoma duodenale* in an urban community in Ogun State. Nigeria. African J of Biotechnology. 2003; 2(11), 470-473.
36. George, S., Kaliappan, S.P. et al. Identification of *Ancylostoma ceylanicum* in children from the tribal community in Tamil Nadu, India using a semi-nested PCR-RFLP tool. Trans R Soc Trop Mes Hyg.2015; 109:283-5.
37. Inpankaew, T., Schar, *et al*. High prevalence of *Ancylostoma ceylanicum* hookworm infections in humans, Cambodia, Emerg Infect Dis, 2014; 20:976-82.
38. Beaver, P.C., Jung, R.C., Cupp, E.W. Clinical Parasitology. Philadelphia: Lea and Febiger.1984
39. Hoagland, K.E., Schad, G.A., *Necator americanus* and *Ancylostoma duodenale*: life history parameters and epidemiological implications of two sympatric hookworms of humans. Exp Parasitol. 1978; 44:36-449.