Comparative Evaluation of Harada–Mori and Agar Plate Culture for the Identification of Hookworm Species under Limited Resources

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Abstract

Background: Human hookworm infection has widespread socioeconomic and public health implications. Several coproculture techniques have been developed for morphological identification of hookworm larvae under limited resource availability. The objective of this study was to compare the performances of Harada–Mori culture (HMC), agar plate culture (APC), and modified APC (MAPC) of hookworm positive stool specimen for identification of hookworm species occurring in East Sikkim, India. Materials and Methods: This prospective cohort study was performed using 180 stool specimen collected from children who attended Central Referral Hospital and Sir Thodup Namgyal Memorial Hospital, with the complained of gastrointestinal symptoms. Blood samples were also collected to correlate with the complete blood count (CBC). The hookworm positive stool specimen evaluated by microscopy was subjected to HMC, APC, and MAPC techniques to harvest hookworm larvae. Stoll’s dilution egg count for determining egg intensity and CBC were also performed for the children who were positive for hookworm’s eggs in their stool sample. Results: This study observed a predominance of Necator americanus (75%) over Ancylostoma duodenale (25%). CBC results showed high packed cell volume values in 9/12, low hemoglobin 9/12, and high eosinophil count in all the positive children. Stoll’s dilution egg count showed moderate infection in 66.6%, light and heavy infections in 16.7% of children’s. APC method was superior to HMC and MAPC in culturing and identifying hookworm species. Conclusions: APC was observed to yield better results and was easier to perform in limited resource laboratory setting compare to MAPC or Harada–Mori culture techniques.

Keywords: Coproculture, diagnosis, gastrointestinal, hookworm

Introduction

Hookworm infection is one of the important causes of iron deficiency anemia among children worldwide, which is primarily caused by two hookworm species, namely, Ancylostoma duodenale (835 million) and Necator americanus (135 million). A. duodenale is mainly distributed in Middle East, North Africa, India, Australia, and Europe, whereas N. americanus is more common in the Western Hemisphere, Sub-Saharan, Eastern Asia, and Southeast Asia. In India, N. americanus is predominant in South India and A. duodenale in predominant in North India regions. Approximately 400 million children worldwide are infected with intestinal parasites, which result in anemia, growth retardation, cognitive impairment, increased susceptibility to other infection, and several acute complications. Different species of hookworm differ in their morphology, pathogenesis, life cycle, and clinical features. Therefore, specific identification and differentiation of hookworm species is important public health measure for monitoring the severity of illness and the efficacy of mass and effective treatment. Although the drug of choice is similar for both species of hookworm, the severity of anemia differs depending on the different species and worm load in the intestine. Further, the severity of clinical manifestations also depends on the egg intensity and positive correlation is observed between the infecting hookworm species and the severity of anemia.

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The following characteristic difference between filarial (L.) larva of *A. duodenale* and *N. americanus* can be used to differentiate the species by coproculture (1) size (*A. duodenale* 720 µm, whereas *N. americanus* is 660 µm); (2) cuticle in case of *A. duodenale* is shorter (10 µm), lumen larger, and bounded by two thin chitinous wall, and in *N. americanus*, it is larger (15 µm), lumen is short, and bounded by two thick chitinous wall; (3) there is no gap between the esophagus and intestine in *A. duodenale*, in case of *N. americanus*, there is a gap due to prominent anterior dilatation of the intestinal lumen; and (4) posterior end of the intestine has a refractile body in *A. duodenale*; however, this is absent in *N. americanus*. [4]

Direct microscopy examination of stool sample is a simple and rapid test to diagnose hookworm infection; however, microscopy alone cannot differentiate between the hookworm species and other similar species such as Strongyloides nematodes due to morphological similarities of the egg. [10]

Hence, various coproculture techniques are employed for the morphological characterization of *A. duodenale*, *N. americanus*, and Strongyloides. Although hookworm infection is one of the important causes of anemia among children, studies examining the prevalence of hookworm species in Sikkim region of India are lacking. Hence, this study aimed to detect hookworm infection and hookworm species prevalent in Sikkim region of India. This study also compared Harada–Mori culture, agar plate culture (APC), and modified APC (MAPC) techniques in the recovery of hookworm larvae and morphological characterization of hookworm species. In addition, the burden of egg intensity by Stoll’s dilution egg count was also studied.

**Materials and Methods**

This prospective study was carried out in 180 children who attended Sir Thodup Namgyal Memorial Hospital and Central Referral Hospital located in East Sikkim, India, during May 2015 to May 2016. Children in the age group 0–15 who presented with gastrointestinal symptoms were investigated for intestinal parasitic infection. All stool specimens were collected before radiological studies using barium or the administration of bismuth, mineral oil, and anti diarrheal medication that could interfere with the detection and identification of intestinal parasites. Children above 15 years and children below 15 years without gastrointestinal symptoms were excluded from this study. Written informed consent was obtained from parents/guardians of the children. This study was approved by Institution Ethics Committee. (Sikkim Manipal University).

**Collection and processing**

The children/guardians were given a labeled, leak-proof container with a plastic scoop (Hi-Media) to collect the sample as per the standard procedure outlined by the World Health Organization and was processed within 4 h of collection. Then, the samples were examined microscopically before smears and after formal-ether concentration, technique using saline and iodine wet mount coverslip preparation. Positive samples for hookworm eggs were further subjected to three coproculture methods to obtained rhabditiform larvae for morphological speciation. Stoll’s dilution egg count method (number of egg per gram of stool) to identify the worm burden and intensity of the infection was conducted in all the positive specimens. Complete blood counts (CBCs) were performed using AcTTM5 diff Cap Pierce Hematology Analyzer.

**Stool culture**

About 0.5–1 g of fresh samples that contained hookworm eggs were cultured to the rhabditiform larva at 25°C–28°C by the Harada–Mori culture (HMC) technique.[9] The tube was kept for 7–10 days and checked daily. For APC, approximately 2 grams of sample were used and incubated at 26°C–33°C for 2 days.[11] For MAPC, a canal of 1 cm wide was made and approximately 2 grams of samples were smeared on the agar and incubated at 26°C–33°C for 2–3 days as previously described.[12-14] Quantitative hookworm egg counts were obtained by Stoll’s dilution egg count[11], and results were expressed as 100–500 eggs in feces.

**Statistical analysis**

The statistical analysis was performed using Microsoft Excel sheet. Chi-square test was used to measure the probability of association between the three methods, prevalence rate of infection among the age and gender group. Different variables were summarized using frequency tables.

**Results**

The overall intestinal parasitic infection rate observed in this study was 32.2% (58/180) in which hookworm infection rate was 6.6% (12/180) [Table 1]. Hookworm infection associated with or without other parasites was 41.6% and 58.3%, respectively. The incidence rate of *N. americanus* and *A. duodenale* in this study was 75% and 25%, respectively. Other predominant intestinal parasites observed in this study were giardia cyst (7.7%), *Entamoeba histolytica/Entamoeba dispar* (5.5%), and *Taenia* eggs (5%). Infection rate was lower among female than male children and significantly (P<0.05) higher prevalence of hookworm was observed in the age group of 6–10 [Table 2]. Diarrhea accompanied with dehydration, weakness, fever, and bloating was the most prominent symptoms at presentation (66%), and other symptoms included diarrhea alone (15%), loss of weight and appetite (8%), abdominal pain (7%), dysentery (3%), and constipation (1%). CBC results showed packed cell volume values high in 5% of children’s due to dehydration, 75% of affected children were anemic and all the affected children had high eosinophil count [Table 4].

Coproculture positivity was observed in 4/12 (33.3%), 12/12 (100%), and 8/12 (66%) in Harada–Mori filter paper method, APC, and MAPC, respectively [Table 3]. Filariform larvae of *N. americanus* varied from 500 to 620 µm, while *A. duodenale* varied from 690 to 750 µm. Stoll’s dilution egg count showed light (100–500) in 2/12 (16.7%), moderate (eggs = 600–1000) in 8/12 (66.6%), and heavy infections (>1000) in 2/12 (16.7%) children.
**DISCUSSION AND CONCLUSION**

Microscopic examination of direct stool sample mount showed 6.6% of the children had hookworm eggs [Figure 1]. This finding is consistent with other studies; however, a few studies have reported higher incidence rate (39.1%).[15–18] These previous studies were based on single fecal examination, which possibly may have missed the chances of the actual prevalence rate. Moreover, examination based on microscopy may also miss the hookworm eggs in the event of light infection. The inability of conventional microscopy to differentiate between *N. americanus*, *A. duodenale*, and Strongyloides is also a major limitation. Besides molecular methods, coproculture methods are alternative methods for species differentiation. In this hilly area of Northeastern Sikkim, India, our study showed the major prevalence of *N. americanus* species [Figures 2 and 3]. No mixed infection was observed among hookworm species in this study. Compared to the HMC and MAPC, APC showed significantly higher positive rates. In terms of total time taken to hatch the filariform larva, APC and MAPC took an almost similar time period of around 2–5 days, in some cases, MAPC took only 2 days. However, in HMC, it takes 7–10 days. In other similar previous studies with 10.15% hookworm incidence rate, 11.53% were *A. duodenale* and 88.47% were *N. americanus*. In contrast, some studies have reported the equal prevalence of *A. duodenale* and *N. americanus*. However, all these studies were based on HMC techniques only.[12,16,19,20] The difference observed may be due to geographical variations or probably existence of substrains.

Depending on the status of host hemoglobin, a hookworm burden of 40–160 worms per individuals can lead to hemoglobin level of 11 g/dl.[21] Regarding the intensity of the infection, majority of the children (66.6%) in this study had moderate fecal egg count. The prevalence of anemia was higher in females (46.8%) compared to males (36.1%). Preschool and school children have the greatest physiological demands for iron, and hence are at higher risk of developing anemia. Furthermore, the infection rate was higher among 6–10 years old male children. This might be due to male children playing barefoot on contaminated soil, close contact with household pets, and poor hygiene. The higher intensity of fecal egg count in this study could be due to poor environmental sanitation and poor hygiene despite deworming among the children. However, a larger number of population sampling will be required to validate this in the future.

![Figure 1: Hookworm eggs in saline mount (x40)](image-url)

### Table 1: Intestinal parasites among the children (n=58)

<table>
<thead>
<tr>
<th>Types of parasites</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Taenia</em> species</td>
<td>9 (5)</td>
</tr>
<tr>
<td><em>Ascaris lumbricoides</em></td>
<td>6 (3.3)</td>
</tr>
<tr>
<td><em>Entamoeba histolytica</em></td>
<td>10 (5.5)</td>
</tr>
<tr>
<td><em>Hymenolepis nana</em></td>
<td>2 (1.2)</td>
</tr>
<tr>
<td><em>Giardia intestinalis</em></td>
<td>14 (7.7)</td>
</tr>
<tr>
<td><em>Trichuris trichiura</em></td>
<td>3 (1.7)</td>
</tr>
<tr>
<td><em>Enterobius vermicularis</em></td>
<td>2 (1.2)</td>
</tr>
<tr>
<td>Hookworm</td>
<td>12 (6.6)</td>
</tr>
</tbody>
</table>

### Table 2: Age- and sex-wise distribution in the study population

<table>
<thead>
<tr>
<th>Age group</th>
<th>Total male (%)</th>
<th>Positive male (%)</th>
<th>Total female (%)</th>
<th>Positive female (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-5</td>
<td>12 (6.6)</td>
<td>1 (0.5)</td>
<td>4 (2.3)</td>
<td>-</td>
</tr>
<tr>
<td>6-10</td>
<td>61 (33.8)</td>
<td>5 (2.7)</td>
<td>63 (35)</td>
<td>3 (1.7)</td>
</tr>
<tr>
<td>11-15</td>
<td>24 (13.4)</td>
<td>2 (1.2)</td>
<td>16 (8.9)</td>
<td>1 (0.5)</td>
</tr>
<tr>
<td>Total</td>
<td>97 (53.8)</td>
<td>8 (4.5)</td>
<td>83 (46.2)</td>
<td>4 (2.3)</td>
</tr>
</tbody>
</table>

### Table 3: Comparisons of Harada-Mori culture, Agar plate culture, and modified agar plate culture method

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>HMC (M1)</th>
<th>APC (M2)</th>
<th>MAPC (M3)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of positive (n=12) (%)</td>
<td>4 (33.3)</td>
<td>12 (100)</td>
<td>8 (66.6)</td>
<td>Pair-wise comparison M1:M2 P&lt;0.05; M1:M3 P&lt;0.05; M2:M3 P&lt;0.05</td>
</tr>
<tr>
<td>Time taken to hatch larva (days)</td>
<td>7-10</td>
<td>2-5</td>
<td>2-3</td>
<td></td>
</tr>
</tbody>
</table>

HMC: Harada-Mori culture, APC: Agar plate culture, MAPC: Modified agar plate culture

### Table 4: Results of complete blood count for the children with hookworm infection

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal range</th>
<th>Males (%)</th>
<th>Females (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>&gt;11</td>
<td>1 (8.3)</td>
<td>2 (16.6)</td>
</tr>
<tr>
<td>Hct (%)</td>
<td>&lt;11</td>
<td>7 (58.4)</td>
<td>2 (16.6)</td>
</tr>
<tr>
<td>&lt;36</td>
<td>&lt;54</td>
<td>5 (41.6)</td>
<td>4 (33.4)</td>
</tr>
<tr>
<td>&lt;27</td>
<td>&gt;32</td>
<td>1 (8.3)</td>
<td>1 (8.3)</td>
</tr>
<tr>
<td>&lt;31</td>
<td>&gt;35</td>
<td>2 (16.6)</td>
<td>0</td>
</tr>
<tr>
<td>Eosinophil count</td>
<td>&lt;0.04</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>&gt;0.40</td>
<td>8 (66.6)</td>
<td>4 (33.3)</td>
<td></td>
</tr>
</tbody>
</table>

MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, Hct: Hematocrit
The reduced mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration and increased eosinophil count observed in this study is a reflection of parasitic infection. The hookworm species differ in susceptibility to anthelminthic dosage regimen\textsuperscript{[7,22]} and have differing route of infection (\textit{N. americanus} infection is mainly by skin penetration while \textit{Ancylostoma} spp. infections are common due to ingestion of infective third-stage larvae). Hence, species identification is paramount in designing appropriate and effective prevention and control strategies. Anemia in children is a worldwide public health problem, in which hookworm infection is one of the main causes. \textit{A. duodenale} causes more blood loss compared to \textit{N. americanus}. It has been estimated that a single \textit{A. duodenale} and \textit{N. americanus} worm ingests about 150 µl and 30 µl of blood per day, respectively. Hence, Hb concentration falls as the intensity of infection rises.\textsuperscript{[23]} In this study, 9/12 of the children with the Hookworm infection had low Hb level (<11 g/dl), which may progressively decrease as the hookworm ingests blood. However, did not observe any significance difference between the blood count of children infected with \textit{N. americanus} and \textit{A. duodenale} and will probably need larger sample size to understand this association.

Compare to molecular methods the cultural techniques are easy and less expensive, although time-consuming but are an ideal alternative in a less resource-setting area like Sikkim. Limitation of this study was the use of the single fecal sample which might lose the chances of hookworm infections from the same patients. Coproculture is also time-consuming and needs well experienced staff to differentiate the species. Nevertheless, ordinary APC yielded better results for the isolation of larva from the eggs in the stool and is also easier to perform in any laboratory with minimal resources.

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Nil.

Conflicts of interest
There are no conflicts of interest.

REFERENCES