

Application of Haradi Mori's Culture Method for Differentiating Hookworm Species in Two Major Cities of Southern Nigeria

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Abstract: The study was undertaken to differentiate hookworm species in Owerri and Port Harcourt during late rainy and early dry seasons, by the application of Harada-mori culture methods. Six hundred faecal. Samples (300 in each of the cities) were screened for positivity of the hookworm ova. Prevalence of hookworm infection was statistically higher in Owerri (23.3%) than Port Harcourt (13.3%, $p < 0.005$) and this was related to seasons of study. Sections of the cities with poor drainage and lower standard of hygiene (Diobu, Port Harcourt and Amakohia, Owerri) had higher prevalence rates (8%, 11.6% respectively) than those zones (Main towns) with improved standard of sanitation and adequate drainage facilities (prevalence rates: 1.3 and 3.3%, respectively). Thirty five of the forty positive samples (87.5%) in Port Harcourt revealed the filariform larvae (second larval stage) of *Necator americanus* 25.5% were *Ancylostoma duodenale*. Similarly, 63 sixty three of the seventy positive samples (90%) obtained from Owerri were *Necator americanus* as against 10% which were *A. duodenale*. Both species were harvested from culture of fresh hookworm positive stool samples in Harada-mori apparatus for a period of ten days at room temperature (25-28°C). Predominance of *N. americanus* confirms with previous studies and appears to re-enforce the efficiency of Harada-mori culture method. The method was simple, cost effective and required minimum skills. Relative endemic status of the study areas were highlighted.

Key words: Hookworm species, Port Harcourt, *Ancylostoma duodenale*

INTRODUCTION

Culture is a very vital aspect of parasitology. Although seldom in use in routine laboratory practice, it enhances the study of parasites in relation to pathogenicity, epidemiology, etiology, identification and isolation. In addition, culture is used for detection of latent infections, assessment of drug sensitivity and production of antigen for immunological tests. The cultural practice involves in-vitro breeding of organisms by using aseptic procedures to inoculate them into a medium rich in all basic requirements or essential factors which naturally support their growth. The essential factors may include nutrients, gases, moisture and temperature, which are fundamental for the survival of the organisms in the natural environment.

Ova of two human hookworm species namely *Ancylostoma dicodenale* and *Necator americanus* are practicable distinguishable in stool specimens. However, they had been identified using stool samples by the following methods.

- The Kato-Katz method (Humphries *et al.*, 1997).
- The Enzyme Linked Immunosobent Assay (ELISA) method (Palmer *et al.*, 1966)

- The Genetic marker and ribosomal DNA (R DNA) method (Hawdon 1966, Gasser, *et al.*, 1996 and Romstad, *et al.*, 1998)
- The Harada-mori cultural technique (Parja *et al.*, 1993, Stoltzfus, *et al.*, 1996, Rai, *et al.*, 1997).

These methods has been employed in the differentiation of hookworm species in various parts of the globe. Humpbries *et al.*^[1] used the Kato-Katz method in their studies of the relationship between the use of the human faeces as fertilizer and intensity of hookworm infection in Vietnam. In their studies on response of adult *Necator americanus* to antigens in Zimbabwe, Palmer *et al.*^[2] used the ELISA method. Similarly, Hawdon^[3] made use of the Genetic markers and ribosomal DNA in U.S.A much as Gasser *et al.*^[4] and Romstal *et al.*^[5] used the same method in Australia.

The Harada-Mari cultural method which has been successfully used in India^[6-8] appears to be the simplest and most cost-effective of all the methods. Its chief advantage lies in the availability of the affordable apparatus required for the method. More so, its operation does not require a conventional laboratory as the experiment could be set up in any room (provided adequate precaution is taken to prevent human contact

with faeces containing the ova of hookworm) for some days to enable the ova hatch into the first (Rhabdiform) and second (filarform) larvae. The emerging larvae of the two medically important species of hookworm displays contrasting morphology (especially at the head, gut and tail regions) when viewed under the microscope^[9].

Identification of the particular species prevalent in an endemic area is of utmost importance in epidemiological studies in view of differences in the pathogenicity and egg-laying capacities of the two species. For example it is known that a female *Ancylostoma duodenale* lays about twenty thousand eggs per day while *Necator americanus* counter part produces about ten thousand eggs per day^[10]. In contrast,, a single. *A duodenale* ingests about 150 uL (0.15 mL) of human blood daily while one *N. americanus* blood ingest about 30 uL (0.03 mL) of blood within the same period^[11].

Previous works had placed Nigeria as one of the hookworm endemic areas of the world. Gills^[12] reported a high incidence (71%) of hookworm infection in Akufor Ibadan, Southwestern Nigeria. Onwuliri and Imandeh^[13] observed that hookworm ranked second (17.47%) of all the human helminthosis in Plateau state, North Central Nigeria. Alao *et al.*^[14] reported a similar hookworm infection rate (15.5%) in Adamawa state, Northeastern Nigeria. In Western part of Nigeria,

Adenusi^[15] working on distribution of *N. americanus* and *A duodenale* reported a high single infection for *N. americanus* (72.0%), moderate mixed infection (23.4%) for both species and low single infection (4.5%) for *A duodenale*. In Port Harcourt Rivers State the following prevalence rates had been reported for hookworm infection: 28.6%^[16], 33.6%^[17] and 33.80%^[18]. Also in Choba near Port Harcourt, Akpan^[19] noted that *N. americanus* was the dominant human hookworm species (66.9%) as against *A duodenale* (38.1%)

Similarly, Udonsi *et al.*^[20] observed that 88.61% of hookworm infections were due to *N. americanus* while 11.79% were duo to *A duodenale* in Owerri, Southeastern Nigeria. They observed seasonal variations in hookworm prevalences as follows 78.06-81.04% in October 1976 and 1977 and 67.5% in March 1977. The authors are not aware of any study, which involved the use of Harada-mori culture method for the differentiation of *A duodenal* and *N americanus* in any area within the endemic south eastern and riverine communities of Nigeria. Southeastern town of Owerri and coastal town of Port Harcourt were chosen in the study in view of their prominence as centers of economic activities and their cosmopolitan characteristics.

MATERIALS AND METHODS

600 stool samples were collected from six different locations (zones) in Port Harcourt and Owerri.300 of the samples were obtained from three zones in Port Harcourt. The same number of samples were procured from three zones in Owerri. The specimens were collected in clean wide mouth specimen bottles and labeled accordingly. All the samples were analyzed fresh, in hatches as soon as they were collected; none was preserved in the refrigerator or 10% formol-saline as this would kill ova and hinder hatching. They were initially screened for the presence of hookworm Ova using the direct smear method This involved the placing and emulsifying of two pin size stool samples,one on a drop of physiological saline and the other on a drop of iodine at opposite ends of a clean glass slide. The preparations were covered with cover slips and examined under the microscope using ×10 (2/3 mm) and ×40 (1/6 mm) objective lens with the condenser of the microscope sufficiently closed to give a good contrast for the identification of hookworm ova^[10].

Samples, which contain hookworm ova were immediately, cultured while those that could not reveal hookworm ova by this method were subjected to saturated sodium chloride flotation method. In this technique about 0.5 g of stool sample was dissolved and suspended in test tube containing saturated sodium chloride solution (specific gravity 1.200). The solution was sieved through a tea sieve in fresh sodium chloride solution until a meniscus was formed. A clean grease free cover slip was placed over the meniscus and left for 30 min to harvest the ova which had floated and stacked on the coverslip. The covership was carefully removed by a straight upward pull, placed on a slide and examined^[10].

Samples that were negative with this method were discarded while positive ones were cultured immediately using Harada–mori culture method^[6-8] as follows: 0.1 g of faeces containing hookworm ova was placed on the middle portion of a tapering (13x20 mm) strip of filter paper. The filter paper was inserted into a 15 mL test tube 7 mL of distilled water was carefully added into the test tube ensuring that the faeces wasn't soaked or washed into the bottom of the test tube. The test tubes were labeled using the marker tapes and grease pencil) and stood vertically in a test tube rack. The test tubes were sealed using the cello tape and rubber band and incubated at room temperature (25-28°C) for a maximum period of ten days. At the end of the incubation, the tubes were submerged to three quarters of their length into a beaker of water heated at 50°C for 15 min to kill the

infective larvae as a precaution against infection during harvesting. The rubber hand, cello tapes and cellophane were discarded by means of forceps into a disinfectant. The contents of the test tubes were transferred into a conical centrifuge tubes and centrifuged at 1000 r.p.m. for 5 min to sediment the larvae. The supernatant was decanted and the sediment examined for sheathed filariform larvae using $\times 10$ (2/3 mm) objective with the condenser iris closed sufficiently to give good contrast.

The filariform (infective larvae) of the two species were identified using the following distinct morphological characteristics, which allowed for their differentiation.

Ancylostoma duodenale: The tail and head and were blunt. There was no gap between the oesophagus and intestine. The oesophagus did not end in a thistle funnel shape. **Necator americanus:** The tail and head end were blunt. There was no gap between the oesophagus and the intestine. The oesophagus ended in a thistle funnel shape.

RESULTS

Tables 1,2 and Fig. 1,2 summarized our findings. In Port Harcourt, 40 out of the 300 samples (13.33%) examined had hookworm ova. In contrast, 70 out of the 300 (23.3%) samples examined in Owerri were positive for hookworm ova. In both towns, *N. americanus* was the predominant human hookworm species, being found in 35 and 63 of all the positive samples in Port Harcourt and Owerri, respectively (prevalence rates 87.5 and 90%, respectively). In contrast, *A. duodenale* occurred in 5 and 7 of the positive samples, respectively (prevalence rates.

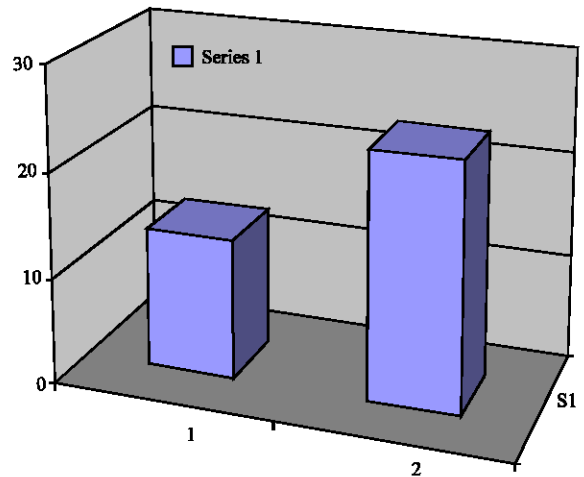


Fig. 1: Overall Prevalence of Hookworm species in Port Harcourt (1) and Owerri (2)

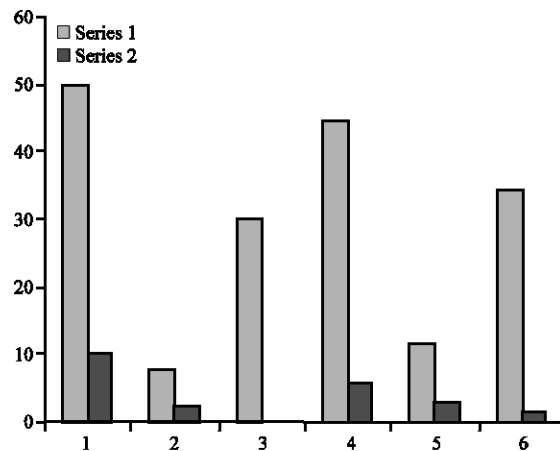


Fig. 2: Distribution of Hookworm (Series 1: Necator americanus; Series 2 Ancylostoma duodenum) in different locations of Port Harcourt (1: Diobu, 2: Main town, 3: Borokiri) and Owerri (4: Amakohia, 5: Main town, 6: Okigwe road)

Table 1: Overall distribution of hookworm infection in port harcourt and owerri

Towns	Zones	No of samples examined	Positive samples	% infection
Port Harcourt	Diobu	100	24	8
	Main town	100	4	1.33
	Borokiri	100	12	4
Total		300	40	13.33
Owerri	Amakohia	100	35	11.6
	Main town	100	10	3.3
	Okigwe road	100	25	8.3
Total		300	70	23.3

Table 2: Distribution of hookworm species in port harcourt and owerri

Hookworm sp.	Port Harcourt			Owerri		
	Diobu	Main town	Borokiri	Amakohia	Main town	Okigwe Road
<i>Necator americanus</i>	20(50%)	3(7.5%)	12(30%)	31(44.3%)	8(11.4%)	24 (34.3%)
<i>Ancylostoma duodenale</i>	4 (10%)	1 (2.5)	0 (0%)	4(5.7%)	2.9%	1(1.4%)
Total	24(60%)	4(10%)	12(30%)	35(50%)	10(14.3%)	25(35.7%)

N/B () = Prevalence rate

12.5 and 10%, respectively). Overall prevalence rates and species rates were statistically higher in Owerri (23.3%) than in Port Harcourt (13.3%, $p < 0.005$). Location (zonal) variations in prevalence rates were observed in both towns. Diobu zone of Port Harcourt had the highest number of positive samples (24 out of 40) much as Amakohia zone of Owerri (35 out of 70). This was followed by Borokiri, Port Harcourt and Okigwe road, Owerri where 12 out of 40 and 25 out of 70 samples were positive for hookworm ova. Least number of positive samples was recorded in the main town zones of the both Owerri and Port Harcourt.

DISCUSSION

The results showed that prevalence of hookworm infections ranged from 13.33% in Port Harcourt to 23.3% in Owerri metropolis. The figure closely resembles those of Alo *et al.*^[4], Kloss^[21] and Onwuliri and Imandeh^[13] who in their separate studies had observed the prevalence of hookworm infections in their study areas to vary from 15.5 to 20%. However it fell short of the figures (28.6-38.1%) given by Amachree^[16-19,22] in their independent studies. The observation placed Owerri and Port Harcourt among the list of Nigeria towns with moderate endemicity for hookworm infections. Others included Benin City, Mid western Nigeria^[23]; Jos environs, Plateau State, North Central Nigeria^[13], Lagos, South Western Nigeria^[15]; Adamawa State, North eastern Nigeria^[14].

Although the reasons for moderate endemicity were not investing, it could probably be related to the cosmopolitan nature and the poor drainage systems observed in course of the project in the study areas. Amakohia zone of Owerri, which recorded the highest positive samples for hookworm ova, was particularly observed to have very poor drainage systems. The gutters were not opened up and the area usually got flooded with water after even the slightest down pour. Besides, sanitary condition was almost at the lowest ebb in Diobu area of Port Harcourt that had the highest record of hookworm positive samples.

Also it was observed that the prevalence rate for hookworm infection was statistically higher in Owerri than Port Harcourt Table 1 and Fig. 1. This was probably due to the fact that the Owerri study was conducted during the dry period of the year (November to January), a period, infection rate was expected to be high. Earlier, Udonsi *et al.*^[20] had reported seasonal variations in hookworm prevalence. According to them 81.04% prevalence rate for *Necator americanus* was observed in October as against 67.5% in March of the same year. The study at Port Harcourt was conducted during the late rainy season (August-October). Apparently, it would seem that higher infection rate with hookworm is associated with dry season.

Necator americanus was the predominant hookworm species in the two study areas of Owerri and Port Harcourt Fig. 2, Table 2. It occurred in more than 80 of the positive cases in the two towns. The observation carried with reports of previous workers. Akpan^[19] in his work on prevalence of human hookworm in choba, near Port Harcourt noted that the prevalence of *Necator americanus* was 66.90% as against 38.1% for *A. duodenale*. Udonsi *et al.*^[20] reported that 88.61% of hookworm infections in Owerri was due to *N. americanus*

while 11.79% was due to *A. duodenale*. Adenusi^[15] observed a high single infection for *Necator americanus* (72.0%) and *A. duodenale* (4.5%). The predominance of *Necator americanus* over *A. duodenale* had also been reported in most parts of tropical Africa^[24]. The health implication of this trend is minimal. This is in appreciation of the fact that *N. americanus* ingests a lesser amount of blood (30 uL or 0.03 mL) per day than *A. duodenale* (0.15 uL or 0.15 mL). In addition, a single female *N. americanus* lays fewer numbers of eggs (ten thousand) per day than *A. duodenale* (twenty thousand).

The efficiency of Harada-mori culture method for harvesting or differentiating hookworm ova cannot be over emphasized. The method was particularly simple, cost-effective, reproducible and requires minimum labour and skill. Hence, it was successfully applied in the present study. It is therefore recommended for field and epidemiological studies in hookworm endemic regions of the world.

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