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Morphological identification of hookworm species in five regions of Cameroon

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Summary

Infections with hookworms (*Necator americanus* and *Ancylostoma duodenale*) remain a major public health problem in low- and middle-income countries. However, the information about the distribution of each species is inaccurate in many countries since their traditional diagnosis is based only on the identification of eggs in stool under a microscope. We aimed to identify the prevalence of hookworm species using morphological stools to identify L3 larvae to gain insights into the distribution of both species in five regions of Cameroon. Samples were collected from schoolchildren in five regions and 34 subdivisions of Cameroon and examined using the Kato-Katz method. We randomly selected a total of 157 samples among hookworm's positive stool samples. They were cultured using the Harada-Mori test-tube technique. The morphological identification of a total of 8063 isolated hookworm filariform larvae L3 was conducted following established criteria. The sensitivity rate to the Harada-Mori technique was 58 %. Among the 8063 L3 larvae identified during this study, 230 (2.95 %) of L3 larvae were identified as *A. duodenale,* and 7833 (97.15 %) of L3 larvae were identified as *N. americanus*. *A. duodenale* was observed only in the Mouanko subdivision in the Littoral region. The complementary use of the Kato Katz and the Harada-Mori culture techniques to screen hookworm infections contributes to the differentiation of *N. americanus* and *A. duodenale* as the two hookworm species in Cameroon. An extended molecular study in the localities where only *N. americanus* has been identified is necessary to reach more conclusions on the distribution of hookworm species in Cameroon.

Keywords: *Ancylostoma duodenale*; *Necator americanus*; hookworms; Harada Mori culture technique; morphological identification; Cameroon

Introduction

The soil-transmitted helminths (STH) are a group of parasitic nematode worms causing human infection through contact with parasite eggs or larvae that thrive in tropical and subtropical countries' warm and moist soil, but also in other climatic zones. Three main STH invasions, ascariasis, trichuriasis, and hookworms, are considered together because they are common for individuals. Hookworm invasions are currently considered one of the most underfunded neglected tropical diseases (NTDs). According to WHO's recent estimates, more than 1.5 billion people, or 24 % of the world's population, are infected with soil-transmitted helminth worldwide. Over 260 million preschool-age children, 654 million school-age children,108 million adolescent girls and 138.8

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million pregnant and lactating women living in areas where these parasites are intensively transmitted require treatment and preventive interventions (WHO, 2022). The burden of disease from soil-transmitted helminths is primarily attributable to their insidious and chronic impact on the health and quality of life of those infected rather than the mortality they cause. High-intensity infections caused the degradation of nutritional status and cognitive processes and were responsible for some 1.9 million disability-adjusted life years (DALYs) lost in 2019 (Levecke *et al*., 2014; Montresor *et al*., 2022). Many people are infected with hookworms without exhibiting symptoms of the disease. Heavy hookworm invasions in humans are associated with pathologic effects, including protein deficiency, heart failure, delayed puberty and mental dullness. Generally, hookworm disease is exhibited in cutaneous, pulmonary, and intestinal phases, most occurring in children and pregnant women (Schmidt *et al*., 2009; Vercruysse *et al*., 2011). Hookworms also cause and contribute to iron deficiency anaemia, which can negatively impact the health of children and women of childbearing age, foetuses, and newborn babies (Christian *et al*., 2004; Bethony *et al*., 2006).

Most diagnoses and research on the epidemiology of human hookworm invasion rely on using a conventional method to detect eggs in stool samples (Tchuem Tchuente *et al*., 2011, 2013). The benefits of this method are mainly technical simplicity and low cost. Although microscopy egg detection is limited and hampered because *Necator americanus* eggs are similar and morphologically indistinguishable from *Ancylostoma spp*., it is still the gold standard technique for rapidly diagnosing hookworms (Hawdon *et al*., 1996). Identifying the particular species prevalent in an endemic area is of utmost importance in epidemiological studies because of differences in the pathogenicity and egg-laying capacities of the two species. A female of *A. duodenale* lays about twenty thousand eggs daily, while *N. americanus* counterpart produces about ten thousand eggs per day (Chesbrough, 1992). Currently, mass treatments with anthelminthic drugs are performed without identifying the causative species of hookworm infections. Given that a clinical manifestation such as the severity of anaemia differs according to the hookworm species involved, a single *A. duodenale* ingests about 0.15 mL of human blood daily while one *N. americanus* ingest about 0.03 mL of blood within the same period (Beaver *et al*., 1984; Chesbrough, 1992). In the human intestine, *A. duodenale* lives for 1 – 3 years and *N. americanus* for 3 – 10 years with a maximum lifespan of 18 years. It has also been reported that *A. duodenale* is capable of entering latency within the host when the environment is unsuitable for its development and can be transmissible by oral, transplacental and lactogenic routes unlike *N. americanus* (Kumar & Pritchard, 1992; Brooker *et al*., 2004). In addition, diagnosis by precise identification and differentiation of species involved is essential in monitoring the efficacy of mass treatment and effective control of hookworm invasion (Akpan & Agida, 2013; Ngui *et al*., 2012). They can be easily differentiated by using copro-culture diagnostic tools as the stool culture followed by the morphological identification of infective third filariform (L3) larvae or by using molecular techniques such as PCR, PCR-RFLP, and qPCR developed for molecular identification of STH species (Traub *et al*., 2004; Bethony *et al*., 2006; Areekul *et al*., 2010; George *et al*., 2015; Ng-Nguyen *et al*., 2015).

In Cameroon, most studies did not attempt to differentiate and relied on past epidemiological data, which indicates the predominance of *N. americanus* infections (Brooker *et al*., 2000; Tchuem Tchuente *et al*., 2003). Recent studies based on the identification of L3 larvae recovered from the stool of school children after culture showed the occurrence of *A. duodenale* and mixed infections with *N. americanus* in one of the ten regions of Cameroon (Kamwa, 2012). Similar observations were made in neighbouring Nigeria, where *N. americanus* was previously described as the only endemic species, but a study on the morphological identification of L3 larvae allows to describe *A. duodenale* (Adenusi, 1997; Adenusi & Ogunyomi, 2003). Therefore, this study aimed to determine the distribution of the two hookworm species in five regions of Cameroon.

Material and Methods

Study area

Cameroon is divided into two major climatic regions: the northern half falls in the tropical climatic zones, and the southern half in the equatorial climatic zone. The country is divided into ten adminis-

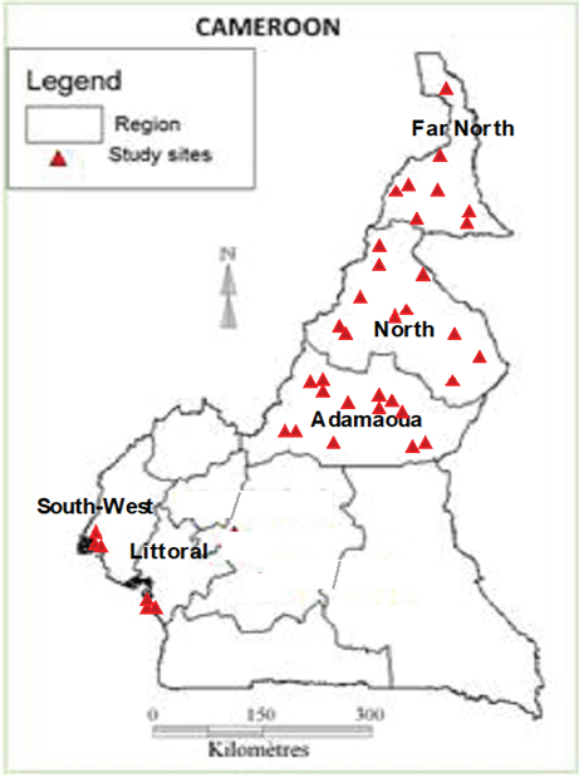

Fig. 1. Map of schools investigated for soil-transmitted helminths (STH) infections.

trative regions: Adamawa, Centre, East, Far-North, Littoral, North, South, South-west, West, and North-west. The tropical climatic zone includes three regions: Far-North, North, and most of Adamawa. In that zone, the rainy season is 4 to 6 months, followed by a long dry season with high-temperature levels. The equatorial climatic zone includes seven other regions characterised by abundant rainfall. STH is widely distributed throughout the country, but the highest prevalence and intensity were observed in the southern half (Tchuem Tchuente *et al*., 2011, 2013). Our investigations were carried out in Ekondo-Titi health district in the southwest region, where the highest prevalence and intensity of hookworm are observed and throughout the northern half during a mapping survey of schistosomiasis and STH activities conducted in the year 2011 – 2012 (Tchuem Tchuente *et al*., 2013). A total of 275 schools were investigated during our study; the distribution of schools is illustrated in Figure 1.

Sampling and data collection

Samples were collected from school children during mapping investigations in 2011 – 2012 and during the survey conducted to assess the efficacy of Mebendazole in 2017. The results of the studies on drug efficacy and mapping have been published elsewhere (Tchuem Tchuente *et al*., 2011, 2013; Montresor *et al*., 2022). Schools were purposely selected in each health district. Children willing to participate were registered, and stool samples were collected from approximately 50 children per school in 60 mL sterile plastic screw-cap vials and transported to the laboratory for examination.

Laboratory Processing and morphological identification

Collection of positive hookworm stool samples

Stool samples were initially screened within 24 hours for hookworm ova by a single Kato-Katz or a Mc Master technique. According to WHO recommendations, the Kato-Katz was performed using the 41.7mg template (WHO, 2012). As described by Levecke *et al*. (2011), the Mac Master method was performed as the standard procedure: 2 g of faeces were filtered and homogenised with 30 ml of saturated saline. Two flotation chambers (1 mL each) were filled for each sample, and three minutes were needed for the eggs to float.

Harada-Mori method

Positive samples that contained at least 48 EPG from the Kato-Katz or 150 EPG from the Mc Master technique were cultured on-site or in the laboratory of the Centre for Schistosomiasis and Parasitology in Yaounde within 24h following sample collection to prevent early hatching of larvae. We proceeded with the Harada-Mori method as follows: about 0.5 g of faeces containing hookworm eggs were placed on the two-thirds portion of a tapering strip of filter paper. The filter paper was introduced into a 15 mL labelled test tube containing 4 mL of distilled water. Test tubes were sealed using paper film, stood vertically in the test tube rack, and incubated at room temperature for a maximum of seven days (Harada & Mori, 1955). Samples were transported to the Centre for Schistosomiasis and Parasitology laboratory in Yaounde the next day due to the lack of equipment necessary for on-site identification. Three to five test tubes were prepared for each sample according to the intensity of infections to increase the number of obtained L3 larvae. At the end of the incubation, the filter paper was rinsed and discarded in alcohol, and the contents of the test tubes were transferred into conical centrifuged tubes and centrifuged at 1500 rpm for 5 min to sediment the larvae. The supernatant was decanted, and the sediment was fixed on a slide with a drop of Lugol and examined for sheathes filariform larvae using x100 and x400 microscope magnification.

Identification keys

The filariform hookworm species were identified according to the WHO morphological identification keys (Table 1) (Yoshida, 1966; WHO, 1981).

Ethical Approval and/or Informed Consent

The protocol of this study was approved, and ethical clearances were obtained from the National Ethics Committee of Cameroon (N° 082/CNE/DNM/09, N° 147/CNE/DNM11). Before data collection, the

Table 1. Detailed morphological characteristics of filariform (L3) larvae of hookworm (Yoshida, 1966; WHO, 1981).

Morphological Keys	*A. duodenale*	*N. americanus*
Length	660 microns	590 microns
Mouth	Less visible	660 microns; Appears dark
Sheath	720 microns; Less striated	Visibly striated and more clearly seen around the end of the body
Oesophagus and intestine	¼ body length; no gap between the oesophagus and the intestine	¼ body length; gap between the oesophagus and the intestine
Intestine	The anterior end is narrower in diameter than the oesophageal bulb	The anterior end is as wide as the oesophageal bulb
Tail	Blunt	Sharply pointed

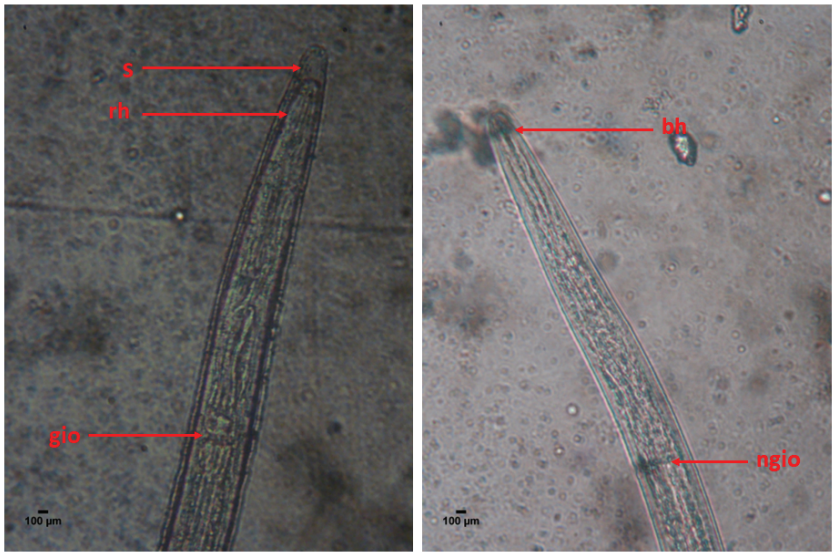

Fig. 2. Head of a hookworm L3 larvae recovered from a hookworm-positive faecal sample

approvals of administrative authorities were obtained. The study's objectives were explained to the school children, parents, and guardians from whom informed consent was obtained and signed.

Results

The identification criteria for hookworm L3 larvae cultured by the Harada-Mori method were based on the distinction of nematode larvae's morphological characters as WHO described (1981). Among the identification keys of nematode larvae's morphological characters, only three were clearly visible on the identical larvae, allowing us to identify the species. These are the mouth, the oesophagus-intestine junction, and the tail morphology. Figures 2 and 3 display some morphological details of L3 filariform larvae observed on hookworm-positive stool samples after culture in our study, showing the differences between *N. americanus* and *A. duodenale*. In *N. americanus,* the sheath around the rounded head, the gap between the intestine and the oesophagus, and the sharply pointed tail with striations are pretty visible. *A. duodenale* is distinguishable from *N. americanus* with the blunt head and tail and the absence of a gap between the intestine and the oesophagus.

From the 275 schools investigated, 157 positive stool samples were collected in 47 schools distributed unequally in the five regions. They were cultured by the Harada-Mori method with an overall sensibility rate of 57 %. The sensibility rate increased as we moved away from hot regions; the highest sensitivity rate was observed in the Littoral (76.47 %) and the South-West regions (71.11 %). Out of 8061 L3 larvae characterized, 230 (2.85 %) were identified as *A. duodenale* and 7831 (97.15 %) as *N. americanus* (Table 2). *A. duodenale* has only been found in the Littoral region, only in the Mouanko health district, where past morphological identification has indicated its occurrence. However, during our study, from the 157 stool samples cultured, we registered 18 cases of *N. americanus* and *A. duodenale* mixed infections (11.46 %) (Fig. 4).

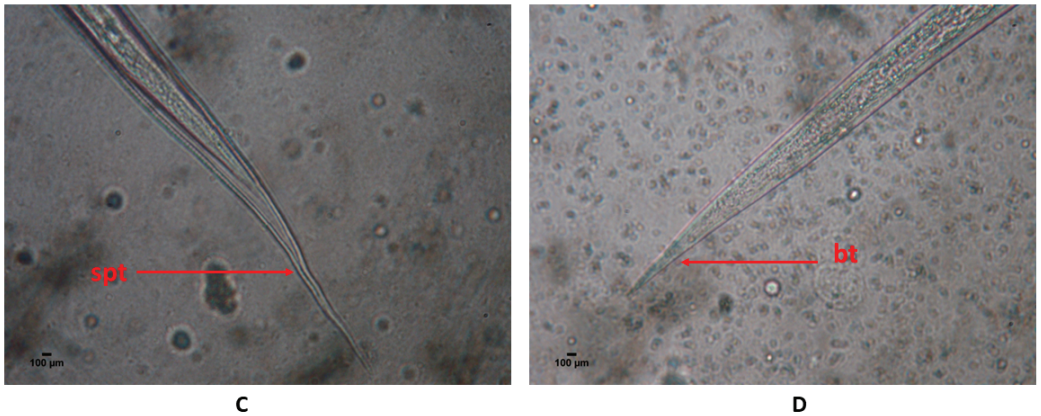

Fig. 3. Tail of a hookworm L3 larvae recovered from a hookworm-positive faecal sample.

Table 2. Number of L3 larvae characterized for hookworm species identification in five regions of Cameroon.

Region	Number of schools investigated	Number of stool samples cultured	Sensibility of Harada-Mori method (%)	Number of L3 larvae identified	
				N. americanus	*A. duodenale*
Adamawa	42	60	68.33	2852	0
North	75	10	40	315	0
Far-North	140	25	32	686	0
South-West	12	45	71.11	3565	0
Littoral	6	17	76.47	413	230
Total	275	157	**57.58**	**7831**	**230**

Discussion

From this study, the results of the morphological identification of hookworm species in five regions of Cameroon using the Harada-Mori stool culture approach show that the sensitivity to the Harada-Mori test differs in the five regions and increases as we move away from the warmer areas. This could be justified by the fact that hookworm species thrive in the optimal temperatures of the larval stages located around 22 – 27°C for *A. duodenale* and 28 – 30 °C for *N. americanus* (Crompton & Whitehead, 1993; Hossain & Bhuiyan, 2016). Following the criteria for species identification described by WHO (1981) and Yoshida (1966), morphological identification of the L3 larvae obtained after stool culture shows the presence of two distinct morphological forms, one belonging to *N. americanus* and the other to *A. duodenale*. The simultaneous presence of both species was observed only in the Littoral region. This result corroborates the findings of Kamwa (2012) in the same locality of Mouanko, where he reported the presence of *N. americanus* (50 %), *A. duodenale* (47.06 %) and cases of mixed infection by both species (14.74 %). In our study, we also registered 11.46 % of mixed infections. Both *A. duodenale* and *N. americanus* infections have been reported in humans in northern Ghana and neighbouring Nigeria after stool culture and identification of the L3 larvae stage (Adenusi & Ogunyomi, 2003; Kwabena, 2009). Our findings also imply that when hookworm infection is diagnosed in the Far-North, North, Adamawa and South-West regions, it is most likely *N. americanus* infection. However, because of some factors, such as the low number of samples collected per site, the lack of suitable equipment for on-site identification, and the temperature variation observed during the transfer of the samples from the site to the laboratory during incubation, the data available cannot be considered sufficient to conclude on the ab-

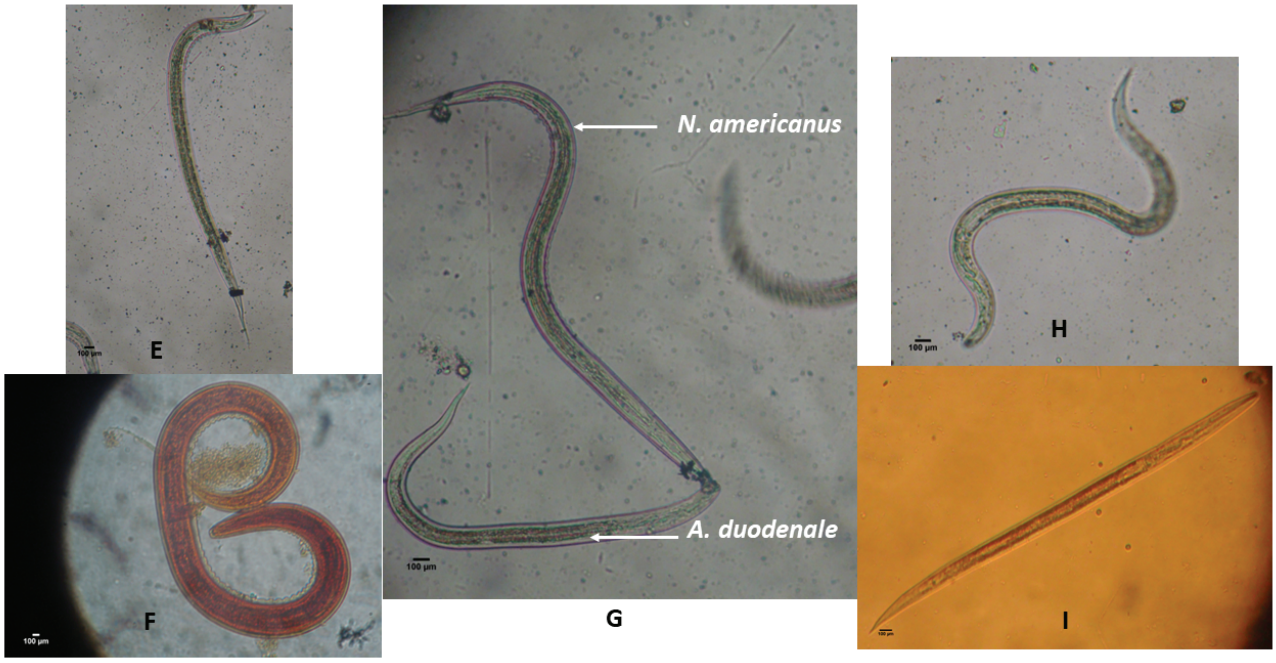

Fig. 4. Hookworm entire L3 larvae recovered from hookworm-positive faecal samples.

sence of *A. duodenale* infection among hookworm-positive school children reporting at these four regions. In addition to these factors, the low yield of the Harada-Mori method compared to other soil-transmitted helminth culture methods, such as the Baermann method and the Agar plate culture technique, could be considered. Indeed, a comparative study of these methods carried out by Reiss *et al*. (2007) showed that the yield of viable larvae from the Agar plate and Baermann methods was comparable (50 % and 47 %, respectively) but greater than that of the Harada-Mori method (2.1 %). Recent work carried out on the sensitivity of the Harada-Mori method compared to the previous method also shows that the sensitivity rate is significantly lower than the sensitivity rates of the Agar plate and Baermann method (Blatt & Cantos, 2003; Nongmaithem *et al*., 2019).

According to the molecular study conducted by George *et al*. (2016), it is not fully understood why *A. duodenale* was not found in the South-West region, especially in Ekondo-Titi, because it was reported to be relatively common in Ekondo-Titi, with a 35 % over 7.5 % prevalence for *N. americanus*. It is possible that our samples were not from the same schools investigated by George *et al*. (2016) or that our data were insufficient to conclude the absence of *A. duodenale*. An extended molecular study in this locality and the localities where only *N. americanus* has been identified will be necessary to conclude the distribution of the different species in Cameroon firmly. Moreover, *A. duodenale* is reported to differ in susceptibility to the same anthelminthic and dosage regimen (Rim *et al*., 1971; Reynoldson *et al*., 1997). Identifying the type of hookworm species being transmitted in a particular community is essential because it influences the burden of iron deficiency anaemia in the community. So, in a community with *Ancylostoma* species, radical treatment and efficient management of anaemia will be required. The difference in susceptibility of the two hookworm species for the same anthelmintic drug mentioned above refers to pyrantel pamoate and bephenium hydroxynaphthoate, two drugs currently out of use in mass deworming campaigns. However, no differential efficacy study has ever been reported on Mebendazole and Albendazole, two drugs currently used in the control of STH and whose efficacy for hookworms has always been lower than that of *Ascaris* (Vercruysse *et al*., 2010; Montresor *et al*., 2022). A subsequent study on this differential efficacy will be conducted.

It is also important to point out that the areas where *A. duodenale* was identified by morphological identification (Mouanko in the Littoral region), and molecular tools (Ekondo-Titi in the South-West region) are near Nigeria's border. The similar geographic conditions in these regions could explain this. There is geographic variance in the distribution of the two human hookworm species, which is a multi-factorial phenomenon, given that human and parasite behavior, ethnicity, climate, temperature, and environmental factors are involved (Hoagland & Schad, 1978). In the northern regions of Cameroon (Far-North, North and Adamawa), the results of the mapping activities show a low prevalence and intensity of the soil-transmitted helminths, but this low endemicity alone could not explain the absence of *A. duodenale* in these regions. A molecular study should be considered to conclude the distribution of hookworms in the northern regions and to establish the role of animal parasites in the endemicity of STH in Cameroon.

Conclusion

Hookworm infection is endemic in all the five regions investigated in our study. The Harada-Mori culture method allowed us to morphologically distinguish two species of hookworms with a predominance for *N. americanus*. *A. duodenale* remains endemic in the health district of Mouanko in the Littoral region. A molecular study is obvious to reach more conclusions on the distribution of these species in Cameroon.

Conflict of Interest

The authors have no potential conflict of interest about this submission to Helminthologia.

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