

Prevalence of Internal Nematode Parasites of Nile Tilapia (*Oreochromis niloticus*) Fish Species caught from Southwestern Part of Lake Tana, Central Gondar, Ethiopia

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ABSTRACT

Background: Nile Tilapia a freshwater cichlid that is indigenous to the Nile basin and one of the world's most important food fishes. Owing to its hardy nature and its wide range of trophic and ecological adaptations and the second most important cultured species. Even though, it is susceptible to the parasite.

Methods: A cross-sectional study was conducted from December 2017 to April 2018 to determine the prevalence of internal nematode parasites of Nile Tilapia (*Oreochromis niloticus*) fish in a southwestern part of Lake Tana, Central Gondar, Ethiopia. The study was performed on a total of 384 *Oreochromis niloticus* fish species which were purposively selected from local fisherman caught using different mesh sized gill nets.

Results: The overall prevalence of internal nematode parasites in this study was 57.3% (220/384). The genera of nematode identified in the body of *Oreochromis niloticus* were *Contraecaecum* the most prevalent 209 (54.4%), the second genus was *Eustrongylides* 7 (1.8%) and the list prevalent was *Camallanus* 4 (1%). The prevalence of nematode was slightly higher in female 196 (58.2%) than male 188 (56.4%) fishes. Similarly, the infection rate in adult and young and in larger and medium fish was higher.

Conclusion: This higher prevalence observed was predominantly related to the distribution of piscivorous birds which are more abundant in an area, where there are high fishing activities and increase the number of discarded filleted wastes. Peoples who have developed the habit of consuming raw fish are at higher risk of becoming infected by the zoonotic nematode parasites. Therefore, awareness creation activities and control of fish parasites should be conducted in the study Lake.

Keywords: *Camallanus*; *Contraecaecum*; *Eustrongylides*; Fish; Lake Tana; Nematode; Nile Tilapia

INTRODUCTION

Fisheries in Ethiopia exclusively depend on inland water bodies for fish supply and commercial production as a low-cost protein source. The country's water bodies have a surface area estimated at 7334 km² of major lakes (Lake Tana, Lake Lugo, Lake Chamo, Lake Awassa and Lake Ziway) and reservoirs, and 275 km² of small water bodies with 7185km of rivers within the country (Janko, 2014). The countries fish potential is estimated at 45,000–51,500 tons per year, according to several sources. Thus, less than 38% of this potential is currently exploited, demonstrating considerable room for further expansion through proper management. The construction of new reservoirs, under-exploited river fisheries and aquaculture are some of the development prospects to this end [1].

In Ethiopia, there are about 168 to 183 different species of fish and 37-57 of them are native to the country. The most common and commercialized fish species in Ethiopia are: *Oreochromis niloticus*, *Clarias gariepinus*, Lates species, *Barbus* species and *Bagnus* species [2]. Over seventy species of tilapia fish have been identified, though global tilapia production has been dominated by three members of the genus *Oreochromis*: Nile tilapia (*Oreochromis niloticus*), Blue tilapia (*Oreochromis aureus*) and Mozambique tilapia (*Oreochromis mossambicus*). Tilapia is a more important and highly valued fish species throughout the world as a food source [3].

Lake Tana is the largest lake in Ethiopia and it harbors unique endemic fish species, as well as the commercially important endemic Nile tilapia subspecies. The lake supports a large fishing

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industry, major fish categories of the lake are *Barbus spp.*, *Clarias gariepinus*, and *Oreochromis niloticus* contribute equally to the caught. *O. niloticus* is most abundant in the shallow littoral zone, while *Clarias gariepinus* and the larger piscivorous *Barbus* species are found mainly in the deeper open water area of the lake. These larger species are mainly exploited by the motorized boat fishery. The caught from the reed boat fishery, confined to the littoral zone, consists mainly of *O. niloticus* and *h* The breeding activity of all major species is associated with the rainy period and increase in Lake water level. During the breeding period, a higher caught rate is observed for *Barbus* species. The shortage of fish storage, distribution and marketing facilities, the limited local market and lack of fishing tradition are major constraints to the development of the fishery. According to the Ethiopian Department of Fisheries and Aquaculture, 1,454 tons of fish are landed each year from Lake Tana at Bahir Dar, which the department estimates are 15% of its sustainable amount. About 70% of the fish species in the lake are endemic [4-6].

In spite of increasing demand for fishes, most people do not focus so much attention on fish health situations like other livestock animals. Fish is also prone to diseases like parasitism. It can also be a good host for parasite multiplication that can be acquired by animals and humans through the ingestion of raw or inadequately cooked fish containing the larvae of the parasite [7]. Nematodes are known as roundworms as they are slender, unsegmented worms and round in cross-section, they are an amazingly abundant and successful animal group, particularly in the aquatic environment. The common nematodes affecting fish are *Camillanus*, *Capillaria*, *Anisakis*, *Contracaecum*, and *Eustrongyloides*. *Camallanus* is thread-like and adult of it normally protrudes from the tilapia fish anus while *Capillaria* is a large roundworm common in the gut of angel fish. *Eustrongyloides* red worm, larvae form is found in fish and uses fish as an intermediate host with the definitive host being the wading bird. It normally encysts in the peritoneum or muscles of fish causing little or no harm [8-10]. *Contracaecum* spp. in the family *Anisakidae* has been found in the pericardial cavity and viscera of tilapia. Larval *Contracaecum* from freshwater fish showing the typical coiled position in the pericardial cavity during postmortem. The larvae of *Anisakis* and *Pseudoterranova* spp. may encyst within the muscles of host fish [11].

Nematode causes an economic threat to the market value of fish, through consumer attitudes towards the presence of these parasites within food products. Infected fillets are rejected and can increase production costs. As a result, there is a great threat to the fish industry causing a fall in production and fish infected by some parasites could be unsuitable for human consumption and raising a lot of public health concerns, particularly in regions where raw or smoked fish are eaten. Some nematodes are zoonotic and include species such as *Eustrongyloides*, *Contracaecum*, *Anguillicola*, *Philometra*, *Skrjabillanus*, and *Anisakis*. Ingestion of uncooked infected fish meat poses a zoonotic threat to humans. Following ingestion, by humans, the larvae are activated by high temperatures, acidic pH and pepsinogen within the stomach and may cause eosinophilic granulomas of the gut. The bulk of research on nematode until very recently focused mainly on marine habitats, ignoring the inland water bodies of most parts of the world. Nematodes from African water bodies, except for South Africa, hardly received any attention to date [12]. The economic loss in the fishing industry and aquaculture and its zoonotic importance caused by nematode

infections is not well known. Investigation of fish parasites and disease in most parts of Ethiopia water bodies are still not giving attention and there are very limited studies concerning the public health impact of endo-parasites in Ethiopia. Therefore the objective of this study is to provide valuable information on the prevalence and associated risk factors of internal nematode parasites of *Oreochromis niloticus* fish species harvested from southwestern part of Lake Tana caught by local fisherman, Central Gondar.

MATERIALS AND METHODS

Study area

The study was conducted in the southwestern part of Lake Tana. Lake Tana is the largest lake in Ethiopia and the source of the Blue Nile. The lake harbors unique endemic cyprinid fish species, as well as the commercially important endemic Nile tilapia subspecies [13]. It located in Amhara region in the north-western Ethiopian highlands, the lake is approximately 84 kilometers long and 66 kilometers wide, with a maximum depth of 15 meters, and an elevation of 1,788 meters. The Lake is fed by the Lesser Abay, Reb and Gumara rivers. Its surface area ranges from 3,000 to 3,500 km², depending on season and rainfall. The lake level has been regulated since the construction of the control weir where the lake discharges into the Blue Nile. Lake Tana water is slightly alkaline of acceptable pH range (pH 6.98 to 9.97). The temperature varies between 16.4°C to 31.3°C [14,15]. The increased soil erosion brings excess sediment load and causes low oxygen content and mud cover on the gravel beds in the inflowing rivers and wetlands and floodplains are converted to agriculture. This habitat degradation seriously affects the recruitment of these endemic species and threatens the biodiversity of Lake. In the Lakes Tana, a fishery is predominantly focused on Nile tilapia and *Barbus* species [16,17].

Study design

A cross-sectional study using a random sampling method was conducted from December 2017 to April 2018 at the southwestern part of Tana, Central Gondar, Ethiopia to determine the prevalence of internal nematode parasites randomly selected *Oreochromis niloticus* (*O. niloticus*) fish species.

Study population

Study animals were a total of 384 *Oreochromis niloticus* (*O. niloticus*) fish species which were purposively selected fish species from local fishermen harvested from the southwestern part of Lake Tana by local fisherman using different mesh sized gill nets for local consumption of Delghi town people. They are maternal mouthbrooders, oral incubation of the eggs and larvae and extensive care is therefore provided almost exclusively by the female [18]. Nile tilapia (*Oreochromis niloticus*) is until recently the most stocked and farmed fish species in Ethiopia. Nile tilapia is an omnivore that feeds on both plankton and aquatic plants. It generally feeds in shallow waters, as harmful gases (such as carbon dioxide, hydrogen sulfide, and ammonia) and temperature fluctuations found in deep waters create problems for the physiology of the fish. The Nile tilapia thrives on the warmer temperatures commonly found in shallow waters compared to the colder environment of the deep lake. Tilapias are the third most farmed fish in the world after carps and salmonids, accounting for 4% of global aquaculture production [19,20].

Sample size determination

The sample size required for this study was determined based on Thrusfield [21], Since there was no previous research done on the prevalence of common internal parasites of *O. niloticus* in this area, 50% expected prevalence, 95% confidence interval, and 5% precision were used to estimate the sample size. As $n = (1.96)^2 \times P_{exp} (1 - P_{exp}) / d^2$; where: n = required sample size, d = absolute precision required ($d = 0.05$), P_{exp} = expected prevalence of the disease. Accordingly, a minimum of 384 fishes was considered in this study.

Sample collection, transport, and handling

The fish sample was collected from local fisherman; they had harvested using different mesh size gill net from the southwestern part of Lake Tana for local consumption to Delghi town people. The collection method was purposively pick up the only tilapia from which other species fish caught by each local fisherman. All samples of fish were evaluated visually and postmortem examination was done using appropriate postmortem kits using standard evisceration technique previously described by Zhokhov et al. [22]. Briefly, the gastrointestinal tract of individual fish specimens was dissected from the anus to the lower jaw using a pair of dissecting scissors. The various organs including stomach, intestine, liver, heart, gallbladder, and gonads were removed and placed in individual sampling bottle and 5% of formalin was added. The sample was immediately transported to University of Gondar Veterinary Parasitology Laboratory.

Determination of sex, length (cm) and weight (g)

The external and internal reproductive structures were used to determine the sex of fish. In tilapia, males are characterized by the presence of genital papillae located on the ventral side, just before the anal fin through which both milt and urine pass; this structure is absent in females, the female possess an oviduct and a urinary pore and also determined by pressing the abdomen the fish for presence of sperms for adult male fish and eggs, for adult female fish. This was later confirmed by the presence and absence of testes and ovaries which was observed during dissection [23]. The standard length (from the tip of snout to the end of the base of the caudal peduncle) and total length (from the tip of the snout to the extreme end of the caudal fin) measured in centimeter using a meter ruler on dissecting board and record [24]. The host fishes were divided into three groups based on their length, smaller (<18 cm), medium (18-28 cm) and larger (>28 cm) to see whether any relationship exists between the length of fish and parasitic infestation. The weight of each fish was measured using Sf 400 digital kitchen weighing scale (The fishes were categorized into four groups based on their weight to determine their age groups: 70-100 g, 110-200 g, 210-300 g and >310 g, fingerling, juvenile, young and adult fish respectively according to Amare et al. and Imam et al. [25,26].

Parasitological examination

First, the external surface of each organ and body cavity of the fish was examined and then cut opened for examination of parasites. All the collected parasites were preserved in 70% ethanol and fixed in glycerin for further identification. Identification of most parasites was made immediately following standard keys in the literature [27,28].

Data analysis

Microsoft Excel 2016 software was used to store the data and data were analyzed using SPSS (Statistical Package for Social Science) version 21. Descriptive statistics and Chi-square (χ^2) was employed used to test independence and dependency of infestation to sex, length (size of fish) and weight (age group). In all the analyses, the confidence level was at 95% and in all cases p-value less than 0.05 ($p < 0.05$) was also considered as statically turn point to look the association of variables.

RESULTS

In this study out of the examined 384 *Oreochromis niloticus* fish, 220 (57.3%) fish were infected with internal nematode. The overall prevalence of internal nematode among sex was 114 (58.2%) on female and 106 (56.4%) on male, respectively. even though, the occurrence of the nematode did not show any statistical significant difference between male and female ($p = 0.724$, at $p > 0.05$) female fish are more affected than male fish (Table 1).

The prevalence of nematode parasites was evaluated based on different body weight (age group) and length (size category). Hence, prevalence of 4 (50%), 37 (53.6%), 127 (57.6%) and 52 (60.5%) were observed from fingerling (70-100 g), juvenile (110-200 g), young (210-300 g) and adult (≥ 300 g) age group, respectively and the difference was found not statistically significant or no parasitic infection variation between size category ($\chi^2 = 0.910$, $p = 0.823$); at $p > 0.05$) (Table 2).

Similarly, the prevalence of nematode parasites was evaluated based on their different body length (size category) with prevalence of 8 (53.3%), 198 (56.9%), and 14 (66.7%) were recorded from >18 cm (small), 18-28 cm (medium), and < 28 cm (large) size categories, respectively. Likewise, the difference between size categories was also found not statistically significant variation ($\chi^2 = 0.873$, $p = 0.646$), at $p > 0.05$) (Table 3) in this study.

In the current study, there were three genera of nematode (*Contracaecum*, *Eustrongylides*, and *Camallanus*) were recovered from the gastrointestinal tract of *Oreochromis niloticus* fish species. From which *Contracaecum* was the most prevalent nematode with the prevalence of 209 (54.4%), in the study area. The second prevalent nematode genera were *Eustrongylides* with the prevalence of 7 (1.8%) and the least encountered nematode genus was *Camallanus* with 4 (1%). The occurrence of each genus of nematode was highly statistically significant variation in the distribution at $p < 0.05$, $p = 0.000$ (Table 4). However, the infection rate in adult and young, in medium and larger fish was higher.

Lastly, the distribution of the individual nematode genera in relation to sex, age group and size category of the infected *Oreochromis niloticus* fish species showed not a statistically significant variation in the distribution of nematodes of specific genera with respect to sex ($\chi^2 = 1.239$, $p = 0.744$): at $p > 0.05$), weight/age group ($\chi^2 = 5.864$, $p = 0.753$), at $p > 0.05$) and length/size category ($\chi^2 = 4.508$, $p = 0.608$), at $p > 0.05$) of fish examined (Table 5). The genus *Contracaecum* was the most prevalent nematode genera affecting *Oreochromis niloticus* with respect to sexes, each age group and size category of fish caught from the south-west part of Lake Tana.

Table 1: prevalence of internal nematode in relation to sex of *Oreochromis niloticus* fish examined caught from south-west of Lake Tana.

Sex	No of fish examined	No of fish infected	Prevalence (%)	X ² (p-value)
Female	196	114	58.2	0.124 (0.724)
Male	188	106	56.4	
Total	384	220	57.3	

Table 2: Prevalence of internal nematode in relation to weight (Age group category) of *Oreochromis niloticus* fish examined caught from south-west of Lake Tana.

Weight(g) (Age group category)	No of fish examined	No of fish infected	Prevalence (%)	X ² (p-value)
70-100 g (Fingerling)	8	4	50	0.910 (0.823)
110-200 g (Juvenile)	69	37	53.6	
210-300 g (Young)	221	127	57.5	
>310 g (Adult)	86	52	60.5	
Total	384	220	57.3	

Table 3: Prevalence of internal nematode in relation to length/size category/ of *Oreochromis niloticus* fish examined caught from south-west of Lake Tana.

Length(cm)/Size category	No of fish examined	No of fish infected	Prevalence (%)	X ² (p-value)
<18 cm (Small)	15	8	53.3%	0.873 (0.646)
18-28 cm (Medium)	348	198	56.9%	
>28 cm (Large)	21	14	66.7%	
Total	384	220	57.3%	

Table 4: Frequency distribution of nematode genera affecting *Oreochromis niloticus* examined caught from south-west of Lake Tana.

Genera of Nematode	No of fish infected	Prevalence (%)	X ² (p-value)
<i>Contracaecum</i>	209	54.4	384.00 (0.000)
<i>Eustrongylides</i>	7	1.8	
<i>Camallanus</i>	4	1	
Total	220	57.3	

Table 5: Association between distributions of nematode genera with sex, age group and size category of the infected *Oreochromis niloticus* caught from south-west of Lake Tana.

Risk Factors	Nematode genera			X ² (p-value)
	<i>Contracaecum</i> (%)	<i>Eustrongylides</i> (%)	<i>Camallanus</i> (%)	
Sex	Female	54.6	2.6	1.239 (0.744)
	Male	54.3	1.1	
	Total	54.4	1.8	
Age group	Fingerling	50	0.00	5.864 (0.753)
	Juvenile	49.3	1.4	
	Young	55.7	1.4	
	Adult	55.8	3.5	
	Total	54.4	1.8	
Size category	Small	53.3	0.00	4.508 (0.608)
	Medium	54.0	2	
	Large	61.9	0.00	
	Total	54.4	1.8	

DISCUSSION

In this study, an overall prevalence rate of internal nematode 220 (57.3%) was recorded in the body of *Oreochromis niloticus* fish caught from south west part of Lake Tana which was higher prevalent compared to the study of Mathenge, in upper Tana River basin, Kenya, in Lake Lugo (Hayke), Northeast Ethiopia [29], in semi-arid reservoirs West Africa, Burkina Faso [30] only nematode in Lake Ziway, Ethiopia [31], Koka reservoir, Ethiopia

with prevalence of nematode 0.9%, 23.6%, 0.39% 9.5%, 8.46% respectively. However, this study somewhat comparable with the study of Busiso [32], 43.8% of the nematode parasites recovered were from Nile tilapia in lower Umguza dam.

This higher prevalence can be related with a large number of aquatic piscivore birds attracted by fish offal avoided by fish supply association and local fishermen. This study revealed that most of the piscivore bird's species are highly infected by parasites The

higher prevalence of internal nematode parasites in the present study was ascribed to different factors which include absence of proper waste disposal and management system in which the fishes were processed and the waste (scraps and gastrointestinal contents) was dumped in to the Lake shoreline, the presence of fish eating and gastrointestinal contents feeder birds, traditional ways of fishing which damaged the phytoplankton or zooplankton of the Lake (feed/weed) that enhanced competition for feed and the use of small sized fish nets which trapped the fingerlings and juveniles and environmental pollution's (climate change. The present study agreed with the study of Daniel Hussien and Gulelat et al., the environment, people dispose off their waste and fecal matters close to the lake which is subsequently washed into the lake thus providing suitable conditions for parasites to thrive. On the other hand, the problem of parasitic infections is associated with feeding behavior of fish. This behavior of the fish could expose it for easy transmission of parasites from invertebrate and fish intermediate hosts. According to the report of Illán [33], Tilapia needs shallow part of the lake because of they are herbivores and they need wetland for the breeding purpose. In the shallow lake part, the existing weeds around the lake offered a good condition for the propagation of the intermediate hosts of nematode, fish-eating birds and the snails. This increases the prevalence of nematode in the study of fish species also related the multiplication of this type of parasite to the more frequent fish contact in shallower water as well as the higher levels of stress during spawning activities which also lead to a weakening in their health status. Alvarez-Pellitero et al. point out that poor environmental quality conditions may have an influence on the presence of the parasite [34].

The most common nematode parasite genera encountered in the present study were *Contracaecum* 209 (54.4%) followed by (*Eustrongylides* 7 (1.8%) and *camallanus* 4 (1%) in *Oreochromis niloticus*, which was high prevalence compared to the study of Daniel Hussien in Lake Ziway, Ethiopia and in lake Lugo (Hayke), which were *Contracaecum* 8.60%, 20% and *Eustrongylides* 0.90%, 2.7% in the gastrointestinal tract of tilapia respectively according to the report of Gulelat et al. 5.4% of *Contracaecum* in Koka reservoir Ethiopia was recorded but, no record of *camallanus* was found in the above studies, The higher prevalence recorded of *contracecum* was attributed to the fact that this parasite has and infests wide range of final and intermediate hosts such as fish-eating birds (cormorants and pelicans) and larval stages are seen in cyprinds (carp and related species), channel catfish and tilapia [35,36] on the other hand, Since *Contracaecum* spp. in tilapia fish may be a parasite of wild fish more than farmed fish. Epidemiological studies pertaining to parasitic infestations of fish in Ethiopia are sporadic and were not well documented. However, Ageze and Menzir [37] reported prevalence rate of 49.5%, 42.5%, respectively, *Contracecum* infestation in Tilapia species in Northeast Ethiopia and Lake Tana, Bahir Dar, Ethiopia which are comparable with prevalence of *Contracecum* in the present study. In addition Zekarias & Yimer [38] in their study at Lake Awassa in Ethiopia, found the nematode *Contracecum* (39.67%) and *Eustrongylides*, which was absent in this study of Mathenge in upper Tana River basin, Kenya. The prevalence of *camallanus* (1.36%) in this study was almost similar with the report of Gulelat et al. in Koka reservoir, Ethiopia.

The prevalence of *Contracaecum* in tilapias from Lake Kyoga [39] was similar to the 11.1% reported from *Oreochromis mossambicus* in reservoirs in India and Zimbabwe [40,41] but, lower than the rate

of 17-25% recorded in the same species in some South African waters [42]. Much higher prevalence rates of 40-50% were found in *O. niloticus* from Lake Tana and its tributaries in Ethiopia [43]. Also in Ethiopia, Yimer et al. [44] noted a prevalence of 15.6% in *O. niloticus*. However, all the above study was under compared with the present study.

The results showed that female fish recorded highly infected with parasites than male fish which is found in agreement with a higher prevalence rate was encountered in female fish than male [45]. The difference in the infection rate of male and female fish recorded in these studies could be attributed to genetic predisposition and differential susceptibility owing to the difference of their physiological condition. Female fish especially gravid ones are susceptible to helminth infections as this physiological state could reduce the resistance of the fish to infection by the parasites [46]. However this study also present in contrast with most researchers which have reported that male fish are usually more infected than female [47-49].

In this study, the prevalence of nematode parasites was also evaluated based on different size categories and weight (age group) but no significant difference between the prevalence and size categories and weight (age group) of fish. This study revealed a higher nematode parasite infestation in the medium-sized (18-28 cm) 56.9% and longest (≥ 28 cm) size category 66.7% and in the young (210-300 g) 57.6% and adult (≥ 300 g) 60.5%. This finding was almost in agreement with the reports of Allumma et al., Amaechi et al. and Bichi et al. [50-52] who stated that larger fishes were heavily parasitized than the smaller ones. The reason for the higher infection rate in adult and young maybe because of the longer duration of time the older fish were exposed to the agents in the environment. This increases their chances of acquiring the parasite infection with time. Similar observation of infection is higher in adult was reported by Amare et al., who noted that longer fish provide greater surface for infection than smaller fish. In addition a higher prevalence and intensity in adult than young tilapia may be due to the accumulation of the worms over a longer period and their larger size which tend to be too big for the piscivorous bird, which will feed on small and medium-sized fish [53,54].

CONCLUSION AND RECOMMENDATIONS

Fish parasitism constitutes a major threat to fish productivity, and the increased demand on fish as a ready and safe source of protein to humans should trigger further studies on fish fauna and their parasites. The observed infection rate was predominantly related to the distribution of piscivorous (pelicans) birds which are abundant in an area where there are high fishing activities and increase the number of discarded filleted wastes. The most common nematode parasite genera encountered in the present study were *Contracaecum*, *Eustrongylides* and *Camallanus* in *Oreochromis niloticus* those generas also identified by another studies in different Lake of Ethiopia. This clearly shows that fish parasites have not attracted the attention of the concerned bodies in the Lake. That is why fish parasites control attempt was not made in the Lake. The finding of the nematode *Contracaecum* and *Eustrongylides* species represents the potential public health risks, as these parasites are recognized to infect humans from consumption of raw or inadequately cooked fish. Therefore, appropriate strategic control measures targeting to

break the life cycle of nematode parasites should be designed and applied with Sanitary measures consideration, and The number of piscivore birds increases so, the relationship between the birds and these parasites should have to be studied.

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CONTRIBUTION OF AUTHORS

Muluken Abiyu conducted the study, and participated in sample collection and data analysis. Gebrekrstos Mekonnen participated in sample collection, data analysis and manuscript preparation. Kidanu Hailay participated in data cleaning and manuscript preparation. All have read and approved the final manuscript.

AUTHORS DECLARATION

The authors have no conflict of interest. The funders had no role in study design, data collection, and analysis, decision to publish, or preparation of the manuscript.

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