Soil-Transmitted Helminth Infections Among Deneba Primary and Junior secondary Schoolchildren, North-central Ethiopia

By

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Abstract

A cross-sectional study was conducted (between December 2015 and January 2016) to estimate the prevalence of soil-transmitted-helminthic infections (STHI) among Deneba primary school children. A total of 384 children were recruited following the systematic-random sampling technique. Of these, 36(9.4%) were found positive for STH with 16(4.2%) *Ascaris lumbricoides*, 14(3.6%) *Hymenolepis nana*, 5(1.3%) *Enterobius vermicularis* and 1(0.3%) *Trichuris trichiura*. Children who do not use latrine (AOR 0.14, 95% CI 0.06-0.32, p 0.000), with no habit of pre-meal hand-washing (AOR 0.20, 95% CI: 0.08-0.49, p 0.00) and who carried dirty materials in their fingernails (AOR 0.40, 95% CI 0.21-0.94 p 0.03) were at significantly higher risk of STHI. The study demonstrated the public health impact of STHI in the study area.

Keywords: schoolchildren, Deneba, soil-transmitted-helminths, prevalence, risk factors

1. Introduction

Soil-transmitted helminths (STH) are a group of worms, mainly nematodes that transmit through ingestion of infective ova or skin penetration by larvae in the soil. STH infections (STHI) are among serious global public health setbacks affecting the poorest and most deprived sections of a population. The most common STH of global importance are *Ascaris lumbricoides*, *Trichuris trichiura*, the hookworms (*Necator americanus/Ancylostoma duodenale*) and *Strongyloides stercoralis* affecting an estimated 5.3 billion people including 1.0 billion school-aged children (Pullan and Brooker 2012). The greatest STH-related public health burden occurs in sub-Saharan Africa (Bethony et al. 2006, Hotez et al. 2008). A recent World Health Organization (WHO) report (WHO 2013) indicates that *A. lumbricoides* alone infected over one billion people followed by *T. trichiura* 795 million and the hookworms 740 million.

Poverty, illiteracy and impoverished health service facility are the major factors behind the increased prevalence of STHI. Lack of awareness of simple health promotion practices is the main contributing factor. Low-level environmental and personal hygiene due to improper disposal of human excreta and animal wastes leads to frequent contamination of food and drinking water and the wider environment. This in turn results in increased prevalence and intensity of STHI. Although STHI are mostly chronic and remain asymptomatic they induce significant morbidity amongst the poorest rural populations. Both pre-school and school-age children and pregnant women are the most vulnerable groups (WHO 2006, Goodman et al. 2007, Al-Mekhafi et al. 2013). The highest prevalence and intensity of infection are usually observed in school-aged children (WHO 2003). Numerous studies covering a diversity of geographic regions worldwide also indicate that for ascaris and trichuris infections maximum worm burdens occur in human populations at 5-10 years of age (Bundy et al. 1995). Although heavy hookworm infections occur in childhood, frequency and intensity commonly remain high in adulthood, even in elderly people (Bethony et al. 2002).

Like in any other sub-Saharan African country, STHI remain among major public health problems in Ethiopia (Tadesse 2005, Mengistu et al. 2007, Dejenie and Asmelash 2010, Tesema et al. 2011, Abera et al. 2013 and Abera and Nibret 2014). According to the Ethiopian federal ministry of health (FMoH 1996) report, more than half million annual visits to the outpatient services of health institutions are due to intestinal parasitic infections. However, this report may be an underestimate, because most of the health institutions lack appropriate diagnostic methods to detect low level parasite burden.

In Ethiopia, a number of surveys have shown that STH are prevalent in varying magnitudes. A study on schoolchildren in Wondogenet, southern Ethiopia, has found the prevalence of *A. lumbricoides* was 83.4% and that of *T. trichiura* 86.4% (Erko and Medhin 2003). Mengistu et al. 2007 reported 83% prevalence of intestinal parasitic infections among urban dwellers in southwest Ethiopia. Mathewos et al. 2014 also reported 66.7% of intestinal parasitic infections in North Gondar. Tekeste et al. 2013 reported 36.8% in northwest Ethiopia and Abera and Nibret 2014 found 44.2% prevalence in Tilili town.

Treatment of schoolchildren for STHI through the school system will help improve their health status, cognitive development and thus academic performance. But, the relative distribution and health impact of STH differs from locality to locality based on environmental/climatic and socio-demographic factors. Thus, site-specific data are necessary to design appropriate strategies for effective control of STHI. In localities like Deneba town, north-central Ethiopia, baseline infomation on STHI is lacking. This study was, therefore, intended to fill in this gap.

2. Objectives

2.1. General objective

The general objective of the study was to estimate the prevalence of STHI and associated risk factors among children of Deneba primary school.

2.2. Specific objectives

i. To detect and identify STHI among children in Deneba primary school and

ii. To assess the associations of environmental or socio-demographic factors with STHI.

3. Literature review

3.1 Global distribution of soil-transmitted helminthes

STHI are among the most common infections worldwide primarily affecting the poorest and most deprived communities in the tropics and subtropics. Important contributors of increased prevalence of these worms include socio-economic status, defecation practices and cultural differences related to personal and food hygiene as well as housing and sewage systems. Most infections are subclinical; but severe complications occur in children who tend to suffer from the highest worm burdens (Stoker et al. 2009). About 1.5 billion people have STHI with the greatest numbers occurring in sub-Saharan Africa, the Americas, China and East Asia (WHO 2016). According to this some WHO document, over 270 million pre-school-age children and over 600 million school-age children live in areas where these parasites intensively transmit, and are in need of treatment and preventive interventions.

Africa is the heaviest STH-burdened continent although reports vary from country to country, and region to region within a country. For instance, 83.3% prevalence was reported in Lagos suburb, southwest Nigeria (Ibidapo and Omolade 2008), 67.3% in Bushulo village, southern Ethiopia (Terfe et al. 2011), 53.5% Arsi Dodota, southeast Ethiopia (Wado 2010), 44.8% in Yenagoa Metropolis, Niger Delta (Bariweni et al. 2014), 86% in Elburgon municipality, Kenya (Mokua et al. 2014) and 33.8% around Mount Cameroon (Ntonifor et al. 2015).

The highest intensity of ascaris and trichuris infections occurs among school-aged children. It is estimated that 173 million and 162 million people are infected in sub-Saharan Africa with ascaris and trichuris, respectively, with 36 million school-aged children infected with ascariasis and 44 million with trichuriasis (Brooker et al. 2006). For both infections the largest number of cases occurs in Nigeria, where co-infections with hookworm are common (Dada-Adegbola et al. 2005). Tens of millions of cases are also found in Ethiopia, Democratic Republic of Congo and the republic of South Africa.

Compared to hookworm, both ascariasis and trichuriasis exhibit a more patchy distribution in sub-Saharan Africa, with the highest prevalence occurring in equatorial central and West Africa, eastern Madagascar, and southeast Africa (Brooker et al. 2006). In contrast to the high rates of ascariasis and trichuriasis in South Africa (Saathoff et al. 2004), hookworm is less common except in KwaZulu-Natal (Mabaso et al. 2004).

Common drugs used are albendazole (*albenza*), mebendazole (*vermox*), or pyrantel pamoate (*antiminth*), tiabendazole (*mintezole*) and niclosamide (*yomesan*) for helminth infections. From an economic point of view, targeted population chemotherapy programs cost half the price of universal ones (O'Lorcain and Holland 2000).

3.2 Species and biology of soil-transmitted helminths

3.2.1 Genus *Ascaris*

A. lumbricoides is the most common human STH. It is an intestinal roundworm and the largest nematode to infect humans. Currently estimates indicate that more than 1.4 billion people are infected by this nematode worldwide (Stoker et al. 2009)

Global ascariasis prevalence reports are quite variable ranging from 1.5% in southern India (Kaliappan et al. 2014) to 67.7% from Lagos Suburb, Nigeria (Ibidapo and Omolade 2008). In between, 3% prevalence was recorded in daycare centers in Iran (Heidaril and Rokni 2003), 30% among school-age children from rural communities in Honduras (Mary-Theresa 2012), 17.7% in Manu jungle in Peru (Miguel et al. 2014), 60.5% in a rural area of Lahore, Pakistan (Hafeez et al. 2003), 21.5% in the Philippines (Fabricant-Dagoc and Tulang 2013), 23.8% in Mount Cameroon (Ntonifor et al. 2015) and 25.1% among pre-school children in Elburgon municipality, Kenya (Mokua et al. 2014).

Ethiopia is the second ascaris high-burden country in sub-Saharan Africa with 26 million people infected covering 15% of the overall burden in the Region (Hotez and Kamath 2009). Ascariasis is found among literally all segments of the Ethiopian society. Its prevalence reaches up to 60.5% in some localities like Chencha in the south (Abossie and Seid 2014), 14.9% in Butajira in the central plateau (Shumbej et al. 2015) and 5.7% in Wukro in the north (Kidane et al. 2014).

The life cycle of *A. lumbricoides* takes about three months. Ascariasis starts, when *A. lumbricoides* eggs are accidentally swallowed (fig. 1). They can be acquired from dirty fingers, water or food that has been contaminated with feces of an infected human. Larva hatch from the eggs, penetrate the intestinal wall and enter the bloodstream. They stop at pulmonary arteries and stay in the lungs for two weeks. They break into the alveoli and travel up to the respiratory system to the throat to be swallowed again. The migration is needed for the larva to develop into adult. Adult worms attach themselves to the intestinal wall ready to mate and survive by eating food digested by the host and live up to 2 years. A female produces about 200,000 microscopic eggs per day that are passed in feces. The eggs fertilize into infective stage within a few weeks in the right conditions in the soil. Unfertilized eggs are not infective. The eggs are very resistant to chemicals, temperatures and other rough condition (Hagel and Giusti 2010).

The diagnosis is usually incidental when the host passes a worm in the stool or vomit. The eggs can be seen in smear of fresh feces examined under a microscope. The eggs have a characteristic shape. They are oval with a thick, mamillated shell and measuring 35-50 in diameter and 40-70 in length (Dold and Holland 2011).

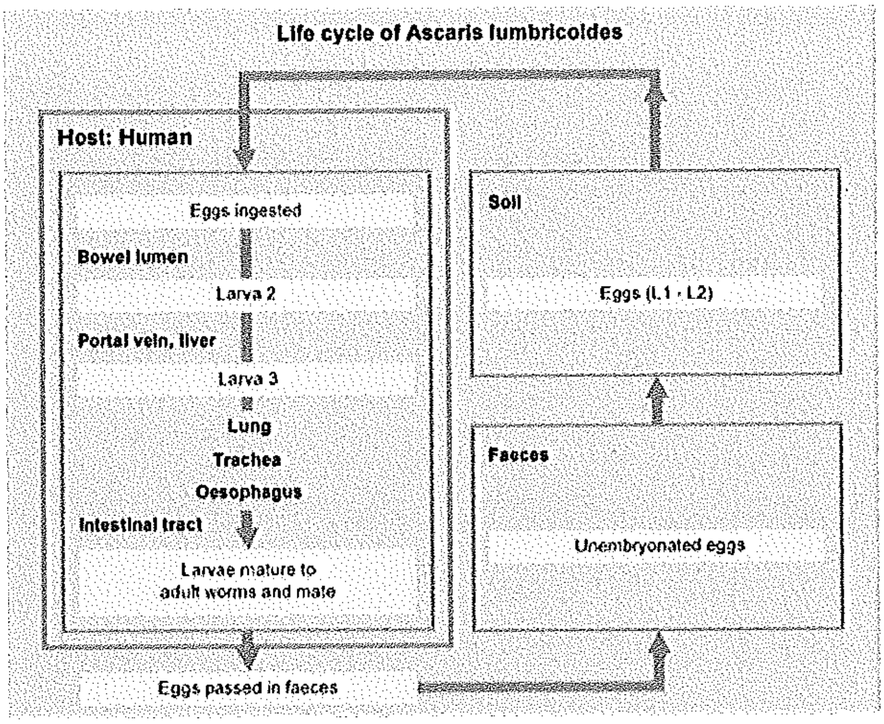

Fig. 1 The life cycle of *A. lumbricoides* (http://www.parasite-diagnosis.ch/ascariasisessential)

3.2.2 Genus *Trichuris*

Members of the genus *Trichuris* are more commonly known as whipworms due to their whip-like adult morphology. This nematode is another dominant STH next to *A. lumbricoides* or the hookworms. It seems to occur in areas particularly where ascaris and hookworms are found because the eggs require the same conditions to embryonate. All the three species can be found in humans together. There are several species within the genus *Trichuris* each infecting specific hosts, but only *T. trichiura* infects man and causes human trichuriasis. It is a parasite that infects many more people than is generally appreciated, up to 800 million people throughout the tropics and temperate regions (WHO 2009).

Like ascariasis, trichuriasis has differing world prevalence report. To mention a few, according to Emmy-Egbe et al. (2012) and Ezeagwuna et al. (2009) 2.6% and 5.8% prevalence of *T. trichiura* was documented, respectively, among schoolchildren in Nigeria. Other workers from Nigeria (Salawu and Ughele 2015) reported 19.5% prevalence in east Nigeria. Mokua et al. (2014) reported 50.8% prevalence of *T. trichiura* among pre-school-age children in Elburgon Municipality, Kenya. Latha et al. (2010) reported 10.8% prevalence of *T. trichiura* infections in schoolchildren in Puducherry, south India. Hafeez et al. (2003) reported 42.1% prevalence of *T. trichiura* in a rural area of Lahore, Pakistan.

In Ethiopia there are 21 million people infected with this parasite, which accounts for 13% of the disease burden in sub-Saharan Africa (Tadesse et al. 2008). *T. trichiura* is reported by different authors from different parts of Ethiopia. Abossie and Seid (2014) reported 9.7% prevalence among primary schoolchildren in Chencha town, south Ethiopia. Mengistu et al. (2007) reported 16.4% prevalence among urban dwellers in southwest Ethiopia and Alemayehu (2008) reported 19.3% prevalence among schoolchildren in Abosa around Lake Zway, south Ethiopia and 6% prevalence was reported by Mathewos et al. (2014) among children in two primary schools around Gondar, northwest Ethiopia.

The total global number of people with *T. trichiura* infection had been estimated to be approximately 1000 million, including 114 million pre-schoolchildren and 233 million schoolchildren (Stephenson et al. 2006).

3.2.3 Hookworm

Hookworm infections in humans are caused by two species, *Necator americanus* and *Ancylostoma duodenale.* They are transmitted through contact with contaminated soil with third stage infective larvae, which either penetrate the skin or when they are ingested in the case of *A. duodenale*. While *N. americanus* has worldwide distribution, *A. duodenale* is more geographically restricted (De Silva et al. 2003).

The adult worms live in the small intestine, attached firmly to the mucous membrane of the gut lining, and feed on blood and tissue. The adult females deposit their eggs in the gut (they can produce up to 20,000 eggs per day); the eggs are then passed out in the feces. The rhabditiform larvae hatch in warm, damp soil (light sandy loam), feeding on bacteria. After about one week during which they have gone through two molts become infective and climb into a suitable position waiting for a suitable host to pass by. The larvae enter the host by penetrating unbroken skin. However it is now recognized that *A. duodenale* can successfully enter man by oral ingestion, this may be more important for this species than skin penetration (WHO 2009). The larvae then enter blood vessels and are carried to the heart, lungs and trachea. They are then swallowed and develop into adult worms in the small intestine (fig. 3). Larvae that are initially swallowed may not show this migration.

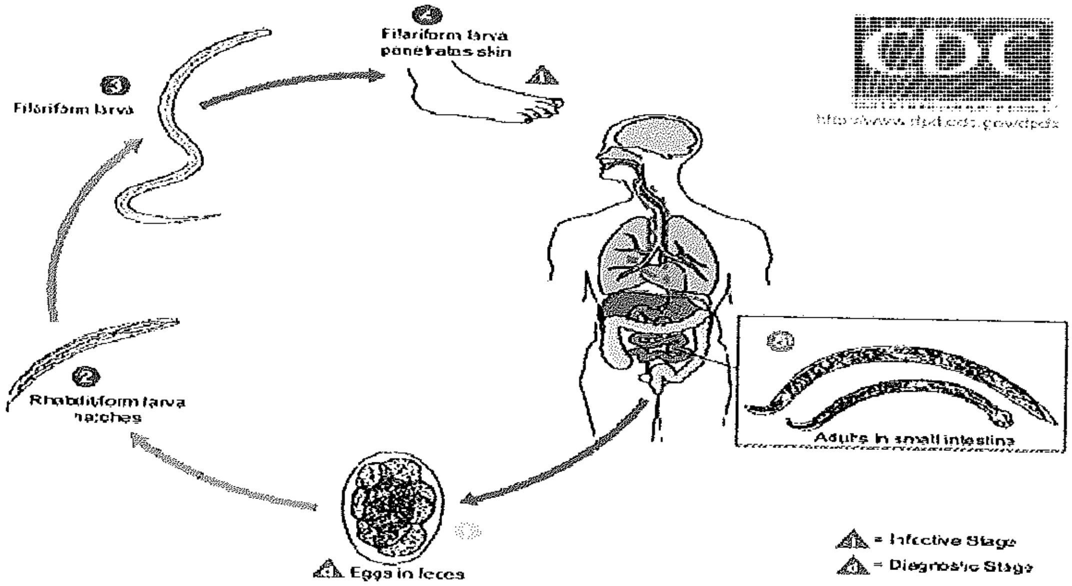

Figure 3 The life cycle of hookworm (http://www.dpd.cdc.gov/dpdx/hookworm/biology.html)

Hookworm diagnosis depends on finding characteristic eggs under microscopic examination of feces, although this is not possible in early infection. The eggs are oval or elliptical, colorless, not bile-stained and with a thin transparent shell membrane.

3.2.4 Genus *Strongyloides* (threadworm)

S. stercoralis is an intestinal nematode commonly found in warm areas, although it is known to survive in the sub-topics (hot and humid conditions). The geographic range of strongyloides infections tend to overlap with that of hookworm due to the eggs requiring the same environmental conditions to induce embryonation. The size and shape of threadworm varies depending on whether it is parasitic or free-living. The parasitic female is larger than the free-living worm (fig. 4). The infective larvae of *S. stercoralis* penetrate the skin of man, enter the venous circulation and pass through the right heart to lungs, where they penetrate into the alveoli. From there, the adolescent parasites ascend to the glottis, are swallowed, and reach the upper part of the small intestine, where they develop into adults. Ovipositing females develop in 28 days from infection. The eggs in the intestinal mucosa hatch and develop into rhabditiform larvae in man. These larvae can penetrate through the mucosa and cycle back into the blood circulation, lung, glottis and duodenum and jejunum; thus they continue the autoinfection cycle. Alternatively, they are passed in the feces, develop into infective filariform larvae and enter another host to complete the direct cycle. If no suitable host is found, the larvae mature into free-living worm and lay eggs in the soil. The eggs hatch in the soil and produce rhabditiform larvae which develop into infective filariform larvae and enter a new host (indirect cycle), or mature into adult worms to repeat the free-living cycle (WHO 2009).

based survey of a rural population of Puducherry (Shivekar et al. 2011), 10.5% among primary schoolchildren in Al-Taameem province, Iraq (Mohammed et al. 1999) and 33.8% among children in daycare centers in Damghan, Iran (Heidari and Rokni 2003).

The prevalence of enterobiasis in Ethiopia was 8.7% in Awramba and neighboring communities in the northwest (Yehenew 2011), 14.1% among schoolchildren in Arsi Dodota (Wado 2010), 1% among primary schoolchildren in Umolante, south Ethiopia (Alemu et al. 2014), 1.4% in Bushulo village, in the south (Terefe et al. 2011), 1.1% among students of Atse Fasil General Elementary School Azezo, in the northwest (Endris et al. 2010), 1.3% among children in selected primary schools in Wukro in the north (Kidane et al. 2014). Dejenie and Asmelash (2010) reported 8.5% prevalence among schoolchildren of different water source users in Tigray, northern Ethiopia.

Infection occurs when embryonated eggs are ingested from the environment, with food or by hand-to-mouth contact. The embryonic larvae hatch in the duodenum and reach adolescence in jejunum and upper ileum. Adult worms descend into lower ileum, cecum and colon and live there for 7-8 weeks. The gravid females, containing more than 10,000 eggs migrate, at night, to the perianal region and deposit their eggs there. Eggs mature in an oxygenated, moist environment and are infectious 3-4 hours later. Direct man-to-man infection and autoinfection are common (WHO 2009).

Humans are the only natural host for the parasite. Infection is facilitated by several factors including overcrowding, wearing soiled clothing, lack of adequate bathing and poor hand hygiene, especially among young school-aged children. Infestation follows ingestion of eggs which usually reach the mouth on soiled hands or contaminated food (fig. 5). Transmission occurs via direct anus to mouth spread from an infected person or via airborne eggs that are in the environment such as contaminated clothing or bed linen. The migration of worms out of the gastrointestinal tract to the anus can cause local perianal irritation and pruritus. Scratching leads to contamination of fingers, especially under fingernails and contributes to autoinfection. Finger sucking and nail biting may be sources of recurrent infection in children (Satoskar et al. 2009).

3.2.6 Genus *Hymenolepis*

Hymenolepiasis is the most common intestinal tapeworm infection of humans caused by *H. nana*. *H. nana* infection has cosmopolitan distribution and most commonly infects humans living under conditions of poor hygiene and poverty (Willms and Sotelo 2001). It is prevalent in school-aged children, particularly most common in children aged 4-10 years, in tropical and subtropical climates of low-income countries (Robert and Tolan 2009).

Unlike other cestodes *H. nana* does not require an intermediate host, although various species of beetles and fleas may serve as intermediate hosts, and infection can occur directly from one infected person to another by fecal-oral route (Mehraj et al. 2008). Humans become infected with *H. nana* by ingestion of water and food contaminated with mouse feces, and can also transmitted from one child to another by passing infective eggs on dirty hands. When eggs are ingested by an arthropod intermediate host, they develop into cysticercoids, which can infect humans or rodents upon ingestion and develop into adults in the small intestine (fig. 6).

When eggs are ingested (in contaminated food or water or from hands contaminated with feces), the oncospheres contained in the eggs are released. The oncospheres (hexacanth larvae) penetrate the intestinal villus and develop into cysticercoid larvae. Upon rupture of the villus, the cysticercoids return to the intestinal lumen, evaginate their scolices, attach to the intestinal mucosa, and develop into adults that reside in the ileal portion of the small intestine and produce gravid proglottids. Eggs are passed in the stool when released from proglottids through the genital atrium or when proglottids disintegrate in the small intestine. An alternate mode of infection consists of internal autoinfection, in which the eggs release their hexacanth embryo, which penetrates the villus, continuing the infective cycle without passage through the external environment. The life span of adult worms is 4-6 weeks, but internal autoinfection allows the infection to persist for years (WHO 2009).

H. nana infections can be diagnosed accurately and rapidly by inspecting the stool for eggs. Preventing fecal contamination of food and water in institutions and crowded areas is of primary importance. General sanitation rodent and insect control (especially control of fleas and grain insects) are also essential for prevention of *H. nana* infection (Donald 2000).

H. nana affects millions of people, worldwide. An estimated the majority of infections are asymptomatic in various regions range from 0.1-58% (Robert and Tolan 2009). It is estimated and is probably associated with a low number of parasites symptoms are vague abdominal distress in light infections, but this can be accompanied by abdominal pain, nausea, vomiting, weight loss and diarrhea.

The prevalence of *H. nana* varies from 2.0% in southeast Nigeria (Nwoke et al. 2015) to 25.8% in Chaco Region, Bolvia Matthy et al. (2011) and 27.6% in Bagh (Azad Kashmir) (Ahmad et al. 2004). Abera et al. (2013) reported 7.4% prevalence of *H. nana* among schoolchildren in Ethiopia, 1% prevalence was reported by Kidane et al. (2014) in Wukro Town and Gelaw et al. (2013) 13.8% prevalence of *H. nana* among schoolchildren at the University of Gondar community school in the northwest.

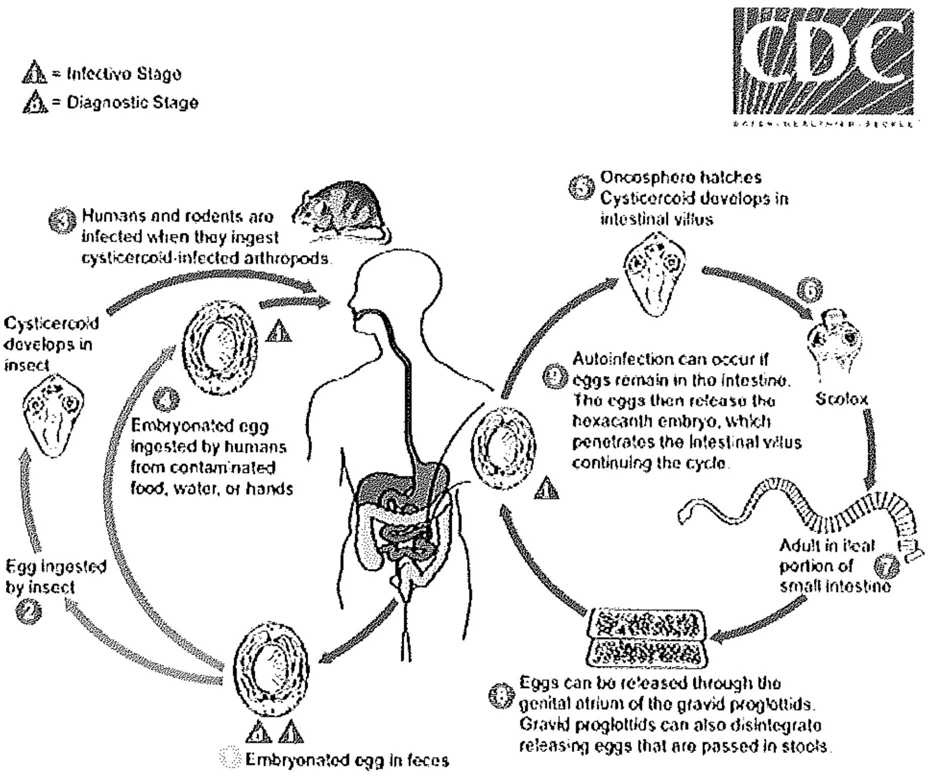

Figure 6 The life cycle of *Hymenolepis nana* (http://www.cdc.gov/dpdx/Hymenolepis *nana*/biology.html)

3.3 Diagnosis of soil-transmitted helminths

For basic diagnosis, specific helminths can be generally identified from the feces and their eggs microscopically examined and enumerated using fecal egg count method (Hall 2000). In the diagnosis of intestinal parasites a wide variety of laboratory methods can be employed (Ahmadi et al. 2007). Stool microscopy using direct wet mounts, formal-ether concentration and the kato-katz techniques offers many relative advantages over other diagnostic methods for detecting intestinal parasites (Bogoch et al. 2006).

Direct saline wet mount provides economical and rapid diagnosis for STH when the intensity of infection is high. It is well established that the direct wet mount technique lacks sensitivity but most laboratories in poor settings still rely on this method for routine stool examinations as a result of its affordability and simplicity (Ukaga et al. 2002, Ahmadi et al. 2007). The formalin ether-concentration method is often used in relatively specialized laboratories. Its relatively lengthy procedure (Lindo et al. 1998) renders it cumbersome with large sample size but has better sensitivity compared to the direct wet mount (Utzinger et al. 2010). The modified Kato-Katz technique (Peters et al. 1980), which is also time-consuming and laborious under field setting, is used to detect helminth eggs. It is specially recommended to detect and quantify schistosomes eggs.

3.4 Risk Factors for soil-transmitted helminths

STH require environments contaminated with egg/larva-carrying feces for transmission. Consequently, they are intimately associated with poor environmental, household/family and personal hygiene. These in turn are determined by level of community education and socio-economy. Uneducated or less educated communities lack basic knowledge of STH transmission and environmental sanitation. Low level of socio-economic development leads to poverty and results in lack of clean water or food, toilet and health facilities. Further, poverty leads to crowded living conditions of large families especially in urban and suburban settings facilitating the transmission of STH. Although STHI are neglected diseases that occur predominantly in rural areas, the social and environmental conditions in many unplanned urban areas are ideal for the persistence of STH.

Moreover; behavioral, occupational and cultural practices as well as institutionalized living conditions such as prisons, daycare centers, orphanages, mental hospitals, etc. may predispose certain sections of a population to increased risk of STHI. Institutionalized living conditions create ideal ground for maintenance of certain types of STH. Pre-school and school-age children who have frequent contact with soil because of their soil-loving behavior and who often put their contaminated hands inside their mouth are more vulnerable. Children's lowest level of personal hygiene is an additional parameter. Changes with age in the average intensity of infection tend to be convex, rising in childhood and declining in adulthood. Farmers and gardeners are other risk-groups with their practices such as use of night soil as a fertilizer further increasing the likelihood of STHI.

Furthermore, immune status which is also a factor of poverty and malnutrition, age as well as presence of other co-infections is additional risk factor for both prevalence and intensity of STHI. Immunocompromised individuals like acquired immunodeficiency syndrome and other chronically ill cases, the elderly, pregnant women and drought hit people are of special concern.

As STH do not reproduce within human hosts high worm burdens, which are the main cause of morbidity, are the result of frequent infections and re-infections within a community. The risk factors allow the persistence of the parasites within a family or community and support their easily perpetuation. Various studies correlated specific risk factors with STHI. Poverty-related household and individual factors such as lack of cement floors, health and hygiene education, potable drinking water, latrines and shoes are implicated (Harhay et al. 2010). As a result, STHI are most prevalent and of high intensity in poor and marginalized rural and urban populations.

In addition environmental (or climatic) factors are other contributors. Climate and topography are crucial determinant of the distribution of helminth parasite infections. STH are highly affected by surface temperature (Brooker et al. 2003), altitude, soil type, and rainfall (Karinki et al. 2004). Adequate warmth and moisture are key features for each of the STH parasites. STH parasite infections exhibit marked seasonality (Brooker and Michael 2000).

4. Materials and methods

4.1 Study area

This study was conducted in Deneba primary school at Deneba town. The town which is 177km to the north of Addis Ababa has an altitude of 1630 meters above sea level. It is the capital of Ensarona Wayu district in North Shewa Zone of Amhara Region (fig. 7). According to the district agricultural and rural development office, the town has a mean annual temperature of 17.5°C. The area receives maximum average annual rainfall of approximately 1600mm from July-August and minimum average rainfall of approximately 1500mm in June and September. The climate is characterized by a long rainy season extending from July to September, a short rainy season that extends from February to March. There is an extended dry season from October to February similar to most other agro-ecological regions of Ethiopia. The type of soil in the study area is clay soil.

Based on the data from the Central Statistical Agency (2013) the town has an estimated total population of 6549, of whom 3505 were males and 3094 females. Majority of the inhabitants are traders and government employees. The rest are involved in different careers.

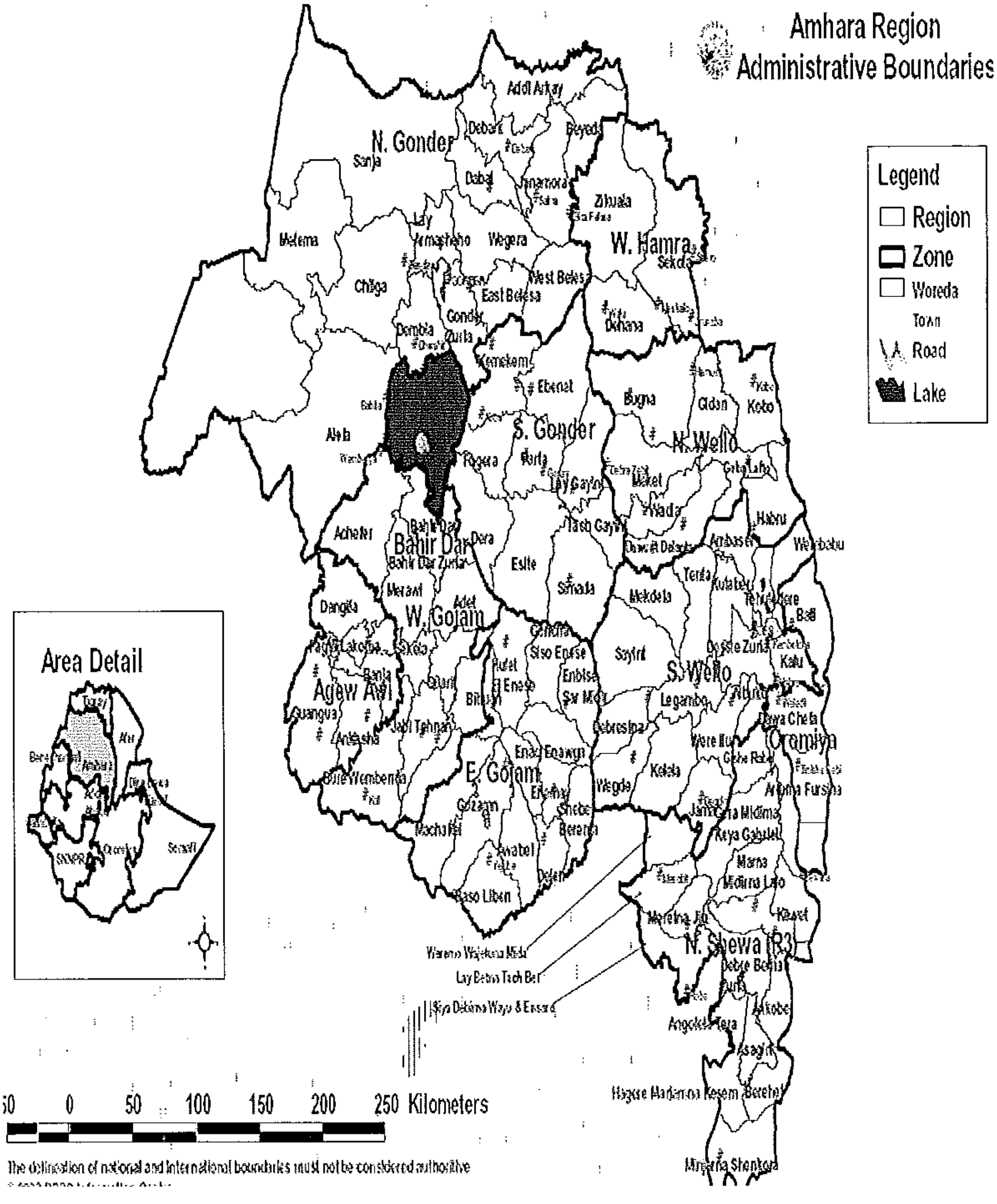

Figure 7 Map of Amhara Region (Source: Hassen 2006)

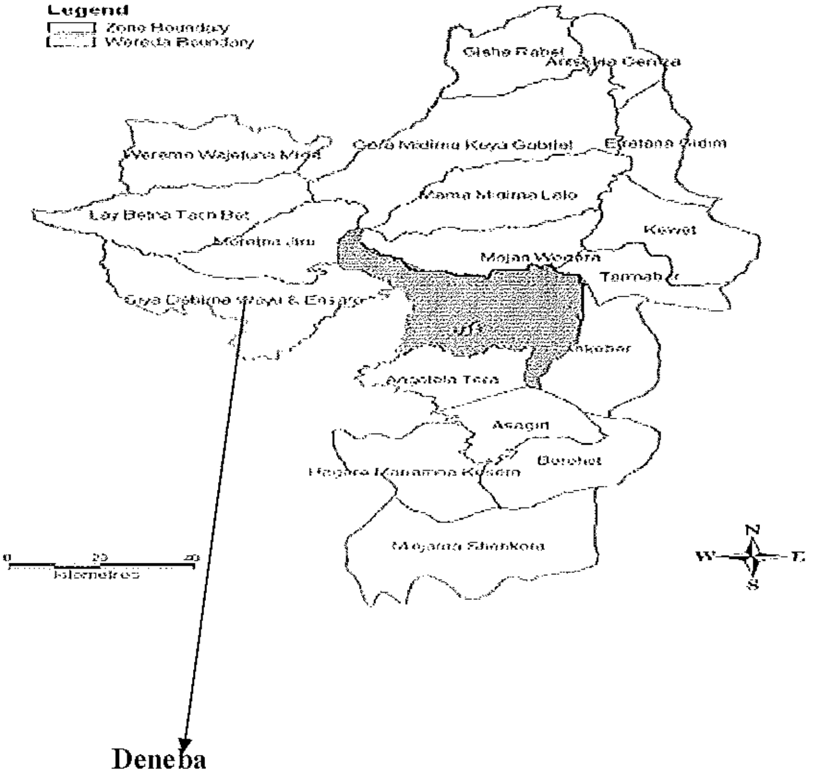

Figure 8 The location of Deneba town in North Shewa Zone (Source: Hassen 2006)

4.2 Study design and population

The study was a cross-sectional parasitological survey conducted on Deneba primary schoolchildren from December 2015-January 2016. School roster of grade 1-8 students was the sampling frame and source population. First the students were categorized into two gender-based strata. Then, children were selected using random sampling technique. Samples were then drawn proportionally from each grade and each classroom from the gender strata.

Since the prevalence rate (p) was unknown in the study area, 50% was assumed, with a marginal error of 5% and 95% confidence interval (CI). For non-response rates, 5% of the sample size was included. The minimum number of the sample size (n) was determined using the statistical formula of sample size calculation (Daniel 1995): $n = z^2p\ (1-p)/d^2$, where n =

the number of the sample size; z = 1.96 at 95% CI; d = margin of error assumed to be 0.05, p = assumed maximum prevalence estimate. Then $n = 1.96^2\ 0.5(1-0.5)/\ (0.05)^2 = 384$.

4.3 Fecal sample collection

Prior to fecal sample collection, socio-demographic characteristics of selected students on parental education level, personal and environmental hygiene, water source, access to latrine in the close vicinity of their homes, and other variables were gathered using a structured questionnaire which was pre-tested by non-study subjects outside the study area. During questionnaire administration the students were carefully visualized for their fingernail status and shoes-wearing habit. The filled out questionnaires were checked for completeness. After a brief instruction on how to collect fecal samples and its amount a labeled disposable plastic cup and spoon were distributed to each student. Children who were treated for STHI for the past 3 months or those who were under treatment for the same at the time of the survey were excluded.

4.4 Analysis of fecal samples

The samples were macroscopically inspected for certain characteristics such as consistency, content, color and presence of adult worms or their fragments before subjecting them to the direct wet-mount procedure. Direct smears were prepared with normal saline for microscopic observation (WHO 1991). About 2g of a stool sample was emulsified with 3-4ml normal saline, and a drop of that was placed on a clean microscopic slide, then a few drops of iodine solution was added and covered with a cover slip. The smear was first examined under 10x objective lenses, then 40 for detailed identification.

4.5 Data analysis

The data was computerized using Excel 2007, cleaned and checked against original document before analysis. All statistical analyses were performed using SPSS 16 statistical package software (SPSS, IBM, Chicago, USA). Univariate logistic regression analysis was used to test the association of independent variables with STHI. All socio-demographic, environmental and life style factors that showed significant associations with STHI and other important parameters with the $p<0.25$ in univariate analysis were selected and entered in multivariate model to identify the most important predictors of STHI. The 95% CI was used to show the accuracy of data analysis. A p-value of <0.05 was considered statistically significant.

4.6 Data quality control

To ensure quality control, before the data collection period, the questionnaire and laboratory materials were pretested. All samples were clearly labeled with the name of students to avoid confusion of their results. Each of the questionnaires was checked whether the necessary information was properly filled. The children were interviewed by using their local language (Amharic) and standard operating procedures were used for specimen collection and processing for maintaining a good quality study.

4.7 Ethical considerations

At the beginning of the study, its objective was explained to the school principal, other concerned local officials, parents and students. Informed consent/assent was obtained from parents/guardians before sample collection. Specimen collection was done using sterile and disposable materials. Individuals diagnosed positive for intestinal parasitic infections were treated free of charge.

5. Results

5.1 Socio-demography

Out of 384(40.04%) students sampled, 186(48.4%) were males and 198(51.6%) females. The study subjects were divided into 3 age-groups (years). The mean age was 11.5 years with minimum and maximum ages 7 and 20 years, respectively. These were 7-10(156(40.6%), 11-14 (165(43%) and 15 and above 63(16.4%). Grades 1-4 students were 193(50.3%) and 5-8 were 191(49.7%).

5.2 Prevalence of STHI

Overall, 36(9.4%) students were positive for STHI with 16(44.4%) males and 20(55.5%) females. Which means among male participants (n=186) the proportion of STHI positives was 8.6% and that of females (n=198) was 10.1%. Highest prevalence of STHI (12.8%) was observed in 7-10 age group and lowest (6.7%) in 11-14. STHI prevalence in urban area 27(11.1%) was greater than rural 9(6.4%). Among grade 1-4 students STHI prevalence was 21(10.9%) and it was 15(7.9%) in 5-8. STHI prevalence differences between sex, age, residence and grade level were not statistically significant (table 1).

Table 1 Prevalence of soil-transmitted helminths in relation to socio-demographic characteristics among Deneba primary and junior secondary schoolchildren, 2015/2016 (N=384)

Variable		Examined (no, %)	Positive (no, %)	p-value
Sex	Male	186(48.4)	16(8.6)	0.62
	Female	198(51.6)	20(10.1)	
Age (year)	7-10	156(40.6)	20(12.8)	0.15
	11-14	165(43)	11(6.7)	
	15 and above	63(16.4)	5(7.9)	
Residence	Urban	244(63.5)	27(11.1)	0.13
	Rural	140(36.5)	9(6.4)	
Grade	1-4	193(50.3)	21(10.9)	0.30
	5-8	191(49.7)	15(7.9)	

The most prevalent worm observed was *A. lumbricoides* (16(4.2%) followed by *H. nana* (14(3.6%), *E. vermicularis* (5(1.3%) and *T. trichiura* (1(0.3%). In age groups 7-10 and 11-14 more females were infected by *A. lumbricoides* (table 2). On the other hand, the prevalence of *H. nana* was more in males of age group 7-10 than the same age group females. The proportion of rural students found positive for *A. lumbricoides* was detected among

(5/140(3.6%) was comparable to that of urban dwellers (11/244 (4.5%). But, concerning *E. vermicularis* relatively higher number of cases (12(4.9%) were from urban than rural (2(1.4%).

Table 2 Distribution of STHI by age and sex among Deneba primary and junior secondary schoolchildren, 2015/2016 (N=384)

STH	Sex	Age			
		7-10(n=156), no.(%)	11-14(n=165), no.(%)	≥15 (n=63), no.(%)	Total (n=384), no.(%)
Al	M	3(3.5)	2(2.9)	1(3.1)	16(4.2)
	F	6(8.5)	3(3.1)	1(3.2)	
Hn					
	M	8(9.4)	0(0.0)	0(0.0)	14(3.6)
	F	3(4.2)	2(2.1)	1(3.2)	
Ev					
	M	0(0.0)	1(1.4)	1(3.1)	5(1.3)
	F	0(0.0)	2(2.1)	1(3.2)	
Tt					
	M	0(0.0)	0(0.0)	0(0.0)	1(0.3)
	F	0(0.0)	1(1.0)	0(0.0)	
Total		20(12.8)	11(6.7)	5(7.9)	36(9.4)

Al: *A. lumbricoides*, Ev: *E. vermicularis*, Hn: *H. nana*, Tt: *T. trichiura*, M: male, F: female, no.: number, STH: soil-transmitted helminths

In univariate logistic regression model as well; sex, age, residence, grade level, and other socio-demographic variables like parent education and job, family size, water source, defecation site, and shoes wearing and its frequency were not significantly associated with STHI-positivity (table 3). On the other hand, habit of latrine use, post-toilet-hand-wash, pre-meal-hand-wash and dirty fingernails were significantly associated with STHI having crude odds ratio (COR), 95% CI and p-values respectively 0.18, 0.08-0.39, 0.00; 4.00, 1.01-15.81, 0.04; 0.26, 0.12-0.58, 0.001 and 0.41, 0.20-0.81, 0.01. The data demonstrate that lack of habits of latrine use, hand washing after latrine and before meal; and fingernail cutting were significant predictors of STHI.

In multivariate analysis (table 4),latrine use, pre-meal-hand-wash and fingernail status were significantly related with prevalence of STHI with adjusted odds ratio (AOR), 95% CI and p-values of 0.14, 0.06-0.32, 0.000; 0.20, 08-0.49, 0.00 and 0.40, 0.21-0.94, 0.03 respectively.

Table 3 Univariate logistic regression analysis for environmental and life style factors associated with soil-transmitted helminths among Deneba primary and junior secondary schoolchildren, 2015/2016

Variable		N	Positive (n, %)	COR	95% CI	p-value
Sex	male	186	16(8.6)	1.19	0.59-2.38	0.62
	female	198	20(10.1)	1.00		
Age (year	7-10	156	20(18.8)	0.59	0.21-1.64	0.31
	11-14	165	11(6.7)	1.21	0.40-3.62	0.74
	≥15	63	5(7.9)	1.00		
Residence	rural	140	9(6.4)	1.81	0.83-3.97	0.14
	urban	244	27(11.1)	1.00		
Grade	1-4	193	21(10.9)	0.69	0.35-1.39	0.31
	5-8	191	15(7.9)	1.00		
Father education	illiterate	110	6(5.5)	0.83	0.16-4.26	0.82
	primary	163	24(14.7)	0.25	0.06-1.22	0.09
	secondary	58	3(5.2)	0.87	0.14-5.46	0.89
	other	9	1(11.11)	0.38	0.03-4.72	0.45
	≥certificate	44	2(4.5)	1.00		
Mother education	illiterate	145	11(7.6)	2.44	0.71-8.39	0.16
	primary	142	14(9.9)	1.83	0.55-6.11	0.33
	secondary	69	7(10.1)	1.77	0.47-6.68	0.39
	other	4	0(0.00)	3.23E8	0.00-0.00	0.99
	≥ certificate	24	4(16.7)	1.00		
Parent job	civil servant	48	5(10.4)	1.00		
	trader	139	18(12.9)	0.78	0.27-2.23	0,65
	farmer	148	9(6.1)	1.79	0.57-5.65	0.32
	labor	30	4(13.3)	0.76	0.19-3.07	0.69
	other	19	0(0.0)	1.88E8	0.00-0.00	0.99
Family size	≥ 4	315	26(8.3)	1.85	0.59-5.75	0.29
	3	41	6(14.6)	0.97	0.25-3.82	0.97
	<3	28	4(14.3)	1.00		
Drinking/cooking water	tap water	254	26(10.2)	1.00		
	stream	1	0(0.0)	0.68	0.31-1.50	0.34
	river	4	1(25.0)	1.25E8	0.00-0.00	1.00
	underground	125	9(7.2)	0.23	0.02-2.47	0.23
Defecation site	latrine	330	22(6.7)	1.00		
	nearby river	11	3(27.3)	0.19	0.05-0.77	0.02*
	shady area	21	2(9.5)	0.68	0.15-3.10	0.62
	open field	20	9(45.0)	0.08	0.03-0.23	0.00*
	other	2	0(0.0)	1.15E8	0.00-0.00	0.9
Shoes	yes	382	36(9.4)	1.00		
	no	2	0(0.0)	1.68	0.00-0.00	0.99
Shoes wearing frequency	always	368	36(9.8)	1.00		
	sometimes	14	0(0.0)	1.75E8	0.00-0.00	0.99
	none	2	0(0.0)	1.75E8	0.00-0.00	0.99
Latrine use	yes	335	22(6.6)	1.00		
	no	49	14(28.6)	0.18	0.08-0.39	0.00*
Post-latrine-hand-wash	no	11	3(27.3)	4.00	1.01-15.81	0.04*
	yes	373	33(8.6)	1.00		
Pre-meal-hand-wash	no	337	25(7.4)	1.00		
	yes	47	11(23.4)	0.26	0.12-0.58	0.001*
Dirty fingernails	no	247	16(6.1)	1.00		
	yes	137	20(14.6)	0.41	0.20-0.81	0.01*

*statistically significant at p-value <0.05, COR: cruds odd ratio, AOR: adjusted odds ratio, CI: confidence interval, n: no of people

Table 4 Multivariate logistic regression analysis of selected risk factors associated with soil-transmitted helminths among Deneba primary and junior secondary schoolchildren, 2015/2016

Risk factors		COR	95% CI	p-value	AOR	95% CI	p-value
Latrine use	Yes	1.00			1.00		
	No	0.18	0.08-0.39	0.00	0.14	0.06-0.32	0.000*
Pre-meal-hand-wash	Yes	0.26	0.12-0.58	0.001	0.20	0.08-0.49	0.00*
	No	1.00			1.00		
Dirty materials in	Yes	0.41	0.20-0.81	0.01	0.40	0.21-0.94	0.03*
fingernails	No	1.00			1.00		

*statistically significant at p-value<0.05, COA: crude odds ratio, AOR: adjusted odds ratio, CI: confidence interval

6. Discussion

While the 9.4% STHI prevalence in this study is nearly comparable with studies on schoolchildren in other settings (Edelduok et al. 2013 (8.1%), Kattula et al. 2014 (7.8%) it is somewhat higher than a recent report from Bolivia (Fabio et al. 2015 (5.9%). On the other hand, the finding is lower than reports by a number of other authors from diverse localities in Ethiopia Tadesse 2005 (27.2%), Dan 2007 (18.4%) Yehenew 2011 (24.5%), Alemu et al. 2014 (26.9%), Hailu and Yimer 2014 (32.0%), Shumbej et al. 2015 (23.3%). The apparent differences might be due to variability in poverty level, awareness, personal and environmental hygiene and overall differences in socio-economic status of the communities under consideration. But, differences in study design and sample size, stool detection techniques, overall human health status and genetics may better explain the observed disparity between the prevalence estimates. In addition, climatic factors such as temperature and rainfall in the study areas may account for the discrepancy.

The prevalence of *A. lumbricoides* (4.2%) in present study was in agreement with Tesema et al. (2011) where 4.4% ascaris cases were detected among patients in Tikur Anbessa hospital. Yimam (2011) recorded ascaris prevalence of 4.2% among children of Tikur Wuha elementary school, Jiga, northwest Ethiopia. Gelaw et al. (2013) found 5.9% among children at the University of Gondar community school and Kidane et al. (2014) 5.7% in selected primary schools in Wukro Town, north Ethiopia. The relatedness of the present figure to that of others from different parts of Ethiopia may demonstrate the widespread and more or less consistent distribution of *A. lumbricoides* cross the country. The prevalence estimates appear declining in recent times probably due to similar improved occasions created by extension health workers in the country including de-worming and other health-related efforts.

Nevertheless striking prevalence data also exist. For instance, 37.2% *A. lumbricoides* prevalence was documented in Ethiopia Terefe et al. (2011). Elsewhere, whereas Latha et al. (2010) recoded *A. lumbricoides* prevalence of 43.2% Ahmad et al. (2004) found as high as 51.7% of his study children harbored the worm. Contrarily very few cases of *A. lumbricoides* (1.9%) were noticed among schoolchildren in Nepal (Sah et al. 2013).

The absence of a significant variation in STHI prevalence between sex-groups in this study disagrees with what was found in Ethiopia (Endris et al. 2010) as well as elsewhere in sub-Saharan Africa (Ezeagwuna et al. 2009). This might be due to the overall lower prevalence estimate in the present study and variations in children's life style, household activity, behavioral, familial or environmental factors. The present study also found no significant association between different age-groups contrary to the findings of some other studies (Uneke et al. 2006, Ezeigbo et al. 2014).

The present result revealed a significant association of STHI with a habit of latrine use in support of the findings of other investigators (Abossie and Sied 2014, Kidane et al. 2014). Of course it is an established knowledge that availability and proper use of latrines is a central strategy of intestinal parasite control. Similarly, the univariate analysis confirmed the association of STHI with post-latrine-hand wash corroborating the result of Abossie and Seid (2014) and Alemayehu (2008). Further, the present study demonstrated that pre-meal-hand-wash significantly reduces the risk of STHI confirming and extending several previous studies (Gelaw et al. 2013, Abossie and Seid 2014, Lemma 2014, Alelign et al. 2015, Kisavi 2014, Shumbej et al. 2015). The similarity indicates the variable in question is properly being practiced and the populations are getting practical benefit from their knowledge and practice to control STH. This study also found that dirty fingernails as significant predictors of STHI agreeing with others (Alemayehu 2008, Sofiana et al. 2011, Abera et al. 2013, Kattula et al. 2014, Shumbej et al. 2015).

Multivariate model of this study significantly associated lack of pre-meal-hand-washing and dirty finger nails independently with a risk of acquiring STHI. The results call for the need to create and enforce increased awareness about personal hygiene. This evidence was produced by numerous similar investigators (Ahmad et al. 2004, Alemayehu 2008, Nmor et al. 2009, Abossie and Seid 2014, Lemma 2014, Kidane et al. 2014), Shumbej et al. 2015).

In this study the method used to identify the parasite was only wet-mount. If the formal-ether concentration and Kato-Katz techniques were used probably more infections might have been recovered.

7. Conclusion

From the total stool samples examined, 9.4% were positive for intestinal helminths. The most frequent intestinal helminths were *A. lumbricoides* followed by *H. nana.* The study showed the public health importance of STHI among children delineating related risk factors.

8. Recommendations

The absence of hookworm and *S. stercoralis*, and very lower presence of *T. trichiura* need to be confirmed in the future using a larger sample size and more than one diagnostic method. On the other hand, the relatively increased prevalence of *H. nana* warrants further study to find out any specific risk factor for this worm.

To minimize the risk of STHI among schoolchildren the following measures worth implementing. Parents require basic knowledge regarding the risk and transmission of STHI. This will help them monitor their children's overall health condition, trim their fingernails and practice pre-meal-hand-washing. Local health personnel and the school administration should cooperate to control the sustained transmission of STHI in the school and beyond. Launching a regular de-worming program is perhaps necessary. If a de-worming scheme is already in place scaling-up other control interventions and integrating them into the scheme can better help control STHI in the area.

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10. Appendix

10.1. Questionnaire

Addis Ababa University, School of Graduate Studies and Department of Zoological Sciences to assess associated risk factors of intestinal soil transmitted helminthes at Deneba primary school children, central Ethiopia.

Subject code _______

1. General information
   1.1 Name of the school ____________________
   1.2 Name of the student ______________
   1.3 Sex ____________________
   1.4 Age ____________________
   1.5 Grade/section of the student ________
   1.6 Address A. Rural B. Urban
   1.7 Religion A. Orthodox B. Muslim C. Catholic D. Protestant E. Other
2. Information on risk factors
   2.1 What is your mother's educational level?
   A. Illiterate B. Primary school educated C. Secondary School educated
   D. Certificated and above E. Others
   2.2 What is your father's educational level?
   A. Illiterate B. Primary school educated C.Secondary School educated
   D. Certificated and above E. Others
   2.3 Do you use latrine? A. Yes B. No
   2.4. If the answer for question number 2.3 is no, where do you defecate and dispose the feces?
   A. Near the river B. Shady area C. Open field D. Others
   2.5. Do you wash your hands after toilet? A. Yes B. No
   2.6. Do you wash your hands before handling and eating food?
   A. Yes B. No
   2.7. From where do you fetch water for drinking and cooking?
   A. From tap water B. From stream C. From river D. Others
   2.8. Is there any dirt in your figure nails (both your right and left figure nails)? Interviewers inspect it.
   A. Yes B. No
   2.9. What is your parent's occupation?
   A. Trader B. Farmer C. Government employee
   D. Manual worker E. Others
   2.10. How many members are there in your family?
   A. Below Three B. Three C. Four D. Five E. Six and above
   2.11. Do you wear shoes?
   A. Yes B. No If no, skips question number 2.12.
   2.12. How often? A. Sometimes B. Regularly
   2.13. Do you eat raw meat? A .yes B. No10.2. Amharic Version of Questione

10.2 *በተማሪዎች የሚሞላ መጠይቅ*

መለያ ኮድ ---------------

ውድ ተማሪዎች :- አፈር ወለድ የአንጀት ጥገኛ ትላትሎች በህብረተሰቡ ላይ በተለይም እድሜአቸው ለትምህር የደረሱ ልጆች ዘንድ ያለውን ስርጭት ለማወቅና ለስርጭቱ በማባስ አጋላጭ ምክንያቶችን ለማወቅና ስርጭቱ የሚገታበትን መንገድ ለመጠቆም ነው፡፡ በተጨማሪም የአንጀት ትላትሎች የሚያደርሱትን ጉዳት ለመቀነስ ይረዳ ዘንድ መጠየቁን እያነበባችሁ በተማኝነትና በቅንንት ትሞሉልኝ ዘንድ በማክበር እጠይቃለሁ፡፡ ለጥናቱ ውጤታማነት ይህ የእናተ እገዛ በጣም አስፈላጊ በመሆኑ ቀና ትብብራችሁን በአክብሮት እጠይቃለሁ፡፡

1. አጠቃላይ መረጃዎች፡
    1.1 የትምህርት ቤቱ ስም --
    1.2 የተማሪው ስም --
    1.3 ጾታ --
    1.4 እድሜ --
    1.5 የክፍል ደረጃና ሴክሽን --------------------------------------
    1.6 የመኖሪያ አድራሻ ሀ/ ገጠር ለ/ ከተማ
    1.7 ሃይማኖት ሀ/ ኦርቶዶክስ ተዋህዶ ለ/ እስልምና ሐ/ ካቶሊክ መ/ ፕሮቴስታንት ሠ/ሌላ
2. ባጋላጭ ሁኔታዎች ላይ የሚሰበሰብ መረጃ
    2.1 የእናት የትምህርት ደረጃ
    ሀ/ ማንበብና መጻፍ የማይችሉ ለ/ አንደኛ ደረጃ ትምህርት የጨረሱ
    ሐ/ የሁለተኛ ደረጃ ትምህርት የጨረሱ መ/ ሰርተፍኬትና ከዛ በላይ ሠ/ ሌላ
    2.2 የአባት የትምህርት ደረጃ
    ሀ/ ማንበብና መጻፍ የማይችሉ ለ/ አንደኛ ደረጃ ትምህርት የጨረሱ
    ሐ/ የሁለተኛ ደረጃ ትምህርት የጨረሱ መ/ ሰርተፍኬትና ከዛ በላይ ሠ/ ሌላ
    2.3 ሽንት ቤት ትጠቀማለህ/ ሽ/
    ሀ/ አዎ ለ/ አልጠቀምም
    2.4 ተራ ቁጥር 2.3 መልስህ /ሽ/ አልጠቀምም ከሆነ አብዛኛውን ጊዜ የምትፀዳዳት የት ነው?
    ሀ/ ወንዝ ዳር ለ/ ጥላ ስር ሐ/ ሜዳ ላይ መ/ሌላ
    2.5 ሽንት ቤት ከተጠቅምህ /ሽ/ በኋላ እጅህን /ሽን/ በሳሙና ትታጠባለህ /ሽህ/?
    ሀ/ አዎ ለ/አልታጠብም
    2.6 እጅህን /ሽን/ በሚገባ ሳትታጠብ/ቢ/ ምግብ ትመገባለህ /ሽ/?
    ሀ/ አልምገብም ለ/ እመገባለሁ
    2.7 ለመጠጥ የሚሆን ውሃ ከየት ታገኛለህ /ሽ/ ?
    ሀ/ ከቧንቧ ለ/ ከምንጭ ሐ/ ከወንዝ መ/ ከጉድጓድ
    2.8 በእጅ ጥፍሮችህ/ሽ/ ላይ ቆሻሻ አለ?
    ሀ/ የለም ለ/አለ
    2.9 የቤተሰብ የስራ ሁኔታ
    ሀ/ ንግድ ለ/ ግብርና ሐ/ የመንግስ ሰራተኛ መ/ የጉልበት ስራ ሠ/ ሌላ
    2.10 የቤተሰብ አባላት ስንት ነው ?
    ሀ/ ከ3 በታች ለ/ ሶስት ሐ/ አራት መ/ አምስት ሠ/ ስድስትና ከዛ በላይ

10.3. Parasitological investigation procedure

The operating procedure for parasitological investigation of Denba primary and junior secondary schoolchildren at a study site.

Direct examination of fecal specimens /wet mount smears preparations procedure

1. Place one drop of 0.85% NaCl on the slide.
2. Take a small amount of fecal specimen and thoroughly emulsify the stool in saline.
3. A drop of emulsified sample placed on a clean microscopic glass slide, and then a few drop of Iodine solution was added.
4. Slide a 22mm cover slip at an angle into the edge of the emulsified fecal drop. Push the cover slip across the drop before allowing it to fall into place.
5. Systematically scan the entire 22mm cover slip with overlapping fields with the 10x objective.
6. Switch to high dry (40x objective) for more detailed study of any suspect egg or larvae.

10.4 Laboratory data collecting format

code	Name of students				Types of parasites											remark
		sex	age	+ve/-ve for parasite	As	Hw	Tt	Ev	H.nana	Strog	Double infect	Triple infect	Multiple infect	Others		

H.nana= *Hymenolepsis nana*
Strong= *Strongyloides stercoralis*
As=*Ascaris lumbricoides*

HW=Hookworm
EV=*Enterobius vermicularis*
Tt=*Trichuris trichiura*

11. Declaration

I, the undersigned, declare that this Thesis is my original work and all source materials are duly acknowledged.

Name Zenebework Wodajneh

Signature.. Date ....