JÖNKÖPING UNIVERSITY
School of Health and Welfare

Prevalence of hookworm infection evaluated with Willis flotation and Formal Ethyl Acetate concentration

- A field study in Da Nang, Vietnam

Main area: Biomedical laboratory science, parasitology
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Sammanfattning

Prevalens av hakmaskinfektion utvärderad med Willis flotation och Formal Etyl Acetat koncentration

- En fält studie utförd i Da Nang, Vietnam

Hakmaskinfektion kan orsaka illamående, magsmärtor och anemi, den mest skadliga effekten ses bland kvinnor i fertil ålder och barn. Infektionen är högfrekvent i fattiga delar av världen med ett stort antal smittade i Asien. Infektionen är många gånger försummad eftersom det sällan orsakar mortalitet, men morbiditeten kan vara destruktiv. I Vietnam är förekomsten av hakmask till stor del okänd, men infektionsfrekvensen tros vara mellan 29-80% i landet. Genom utförandet av en fältstudie i Da Nang, Vietnam, identifierades förekomsten av hakmaskinfektion med hjälp av två metoder, Willis flotation och formal etyl acetat koncentration. Eventuella samband mellan hakmaskinfektion och individernas kön, ålder och geografiskområde utvärderades. Totalt 101 konsekutiva utvalda prover från sjukhus och samhällen i ett ruralt distrikt och tre urbana delar av staden erhölls från både barn, kvinnor och män i åldrarna 1-72. Inga kvantitativa skillnader påträffades mellan de två metoderna och inte heller någon korrelation mellan kön och infektion återfanns (p-värde 0,143). Den totala prevalensen var 16,8%. Den rurala delen av staden visade en högre infektionsfrekvens i motsats till urbana statsdelarna (p-värde 0,001). Individer i åldersgruppen 25-48 uppvisade en högre infektionsfrekvens i motsats till de andra åldersgrupperna (p-värde 0035).

Nyckelord; Soil transmitted helminth, Necator americanus, Ancylostoma duodenale, rurala distrikt, Urbana distrikt.

Summary

Hookworm infection can cause nausea, stomach pain and anemia, with the most harmful effect being found among women of reproductive age and children. The infection rates is high in poor parts of the world with a high number of infected in Asia. The infection is many times neglected since it rarely causes mortality, however the morbidity can be destructive. In Vietnam the prevalence of hookworm is largely unknown, but there is believed to be a 29-80 % infection rate in the country. Through a field study in Da Nang, Vietnam, the prevalence of hookworm was identified using two methods, Willis flotation and formal ethyl acetate concentration. Any correlation between hookworm infection and individuals' gender, age and geographic area was evaluated. A total of 101 consecutive selected samples from hospitals and communities in rural and urban parts of the city were obtained from both gender ranging between 1-72 years in age. No quantitative differences were found between the two methods nor any correlation between genders (p-value 0,143). The overall prevalence was 16,8%. The rural part of the city showed a higher infection rate in contrast to the urban districts (p-value 0,001). Individuals in the age group 25-48 showed a higher infection rate in contrast to the other age groups (p-value 0,035).

Keywords; Soil transmitted helminth, Necator americanus, Ancylostoma duodenale, rural district, urban district.

Table of content

Attachment

Introduction

Soil transmitted helminth (STH) are parasites that spread through soil and live in the gastrointestinal tract (1). There are many types of STHs, one of the most common is hookworm (2), which is classified as a nematode (3). According to the World Health Organization (WHO), STH infections effect humans in the poorest parts of the world (4). Worldwide approximately 1,4 billion people (5) are estimated to be infected by STH and around 900 million are infected by hookworm (6) with the highest number of cases found in Asia. The tropical heat and optimum climate combined with poor and unsanitary conditions increased the risk of the infection (5). Vietnam is one of the developing countries in southeast Asia were hookworm infection frequently occur (5,7).

The hookworm infection can cause a wide range of morbidity, in rare cases the consequences can lead to mortality (3,6). The worm releases thousands of eggs each day which can be seen in faeces (3). To diagnose the infection of hookworm several methods are in use and eggs are detected in light microscope (8). The Willis flotation technique is being used in Da Nang, Vietnam and the Formal ethyl acetate concentration is used in Jönköping, Sweden.

Studies in Northern Vietnam indicate prevalence rates of hookworm infections to be between 50%-80%, (2, 9, 10, 11). A study in central Vietnam, Dai loc district, Quang Nam province showed a 29% infection rate (12), but in many parts of Vietnam the prevalence is largely unknown. For this reason, a scholarship for Minor Field Studies (MFS) was granted in order to do a data-collection in the student-clinic at University of Medical Technology and Pharmacy in Da Nang, Vietnam to investigate the prevalence in the area.

Background

Parasites are organisms that require nourishment from other species in order to survive. They feed on or in a host and benefits at the others expense which in turn can lead to the host being harmed (13). Parasites are divided into several different classes and one of the main groups of parasites are helminths that spread through the soil, known as STH and classified as nematodes. *Ascaris lumbricoides* (roundworm), *Trichuris trichiura* (whipworm), and two species of hookworm *Ancylostoma duodenale* (*A.duodenale*) and *Necator americanus* (*N.americanus*) are STH's and lives within the gastrointestinal tract (14). The first study of human hookworm infection started in the twentieth century, beginning with an Italian pathologists who identified

A.duodenale. The lifecycle of *A.duodenale* was later explained by Looss in 1901 and *Necator americanus* was discovered in the western hemisphere. Hookworm belongs to the family of Ancylostomatidae and adult worms have either teeth for *A.duodenale* or cutting plates for *N.americanus* that line the buccal canal. Another difference between the two species are the geographic distribution. *N.americanus* is most spread worldwide whereas *A.duodenale* has a more limited geographically spread (3), for example in parts of Europe and countries surrounding the Mediterranean (6). Possible transport host for *N.americanus* is pigs, while *A.duodunale* strictly infect humans without the use of a transport host (3,6). The elaborate attachment structures enable them to stay within the host and cause significant gastrointestinal blood loss (3) and leads to microcytic hypochromic anemia (15).

The hookworm infection frequently occurs in Southeast Asia, for example in Vietnam. In the central part of the country, the third largest city Da Nang is located with a population of 879 500. Da Nang is located on the coastline and is divided into six urban districts, three of them are Son Tra-, Lien Chieu-, and Ngu Hanh Son district. Hoa Vang represent the only rural district of the city. (16).

Prevalence

Infection by hookworm are estimated as a high endemic infection throughout the tropics and sub-tropics. The highest number of hookworm cases occur in Asia (5,7). Even though the rate of infections has dropped slightly during the past 20 years' worldwide it still remains at an unacceptable level. In Southeast Asia over 64,5 million people are affected by hookworm (17). In an elderly study from 1998, that took place in Ha Nam, Vietnam a 59% infection rate of hookworm was found (11). A more recent study from the northern parts of Vietnam, showed that women between the ages of 16-45 had a 76% infection rate (9). An additional study from 2003 estimated that the prevalence in northern Vietnam was 52%. (10). In a study from 2008 conducted among woman in Vietnam 78% were infected by hookworm and 6 % had heavy infection (2). In a study from 2013 conducted in the community in Vietnam, Dai loc district, Quang Nam province showed that 29% were infected by hookworm (12), however the overall prevalence is still largely unknown in many of Vietnam's districts (2).

Lifecycle and symptoms by hookworm

The hookworm eggs hatch into larvae, first stage larvae (L1), known as rhabithform within 2448 hours in the soil during optimal conditions with suitable warmth, humidity and shade. (3,14). The larvae feed on bacteria and organic matter in the surrounding environment (18) before they develop into the infective larval stage, known as third stage larvae (L3) (3) within 5-10 days. They survive in the soil for several weeks before penetrating the skin of the human host causing itch and erythema (6,18). The L3 stage, filariform is about 600 microns (µm) in length (3) and it burrows through tissue until it reaches the blood vessels. The larvae migrate through the venoules to superior- or inferior vena cava towards the lungs, causing pneumonitis and eosinophilia (15). Further, by entering the alveolus it migrates to the bronchial tree and trachea, via pharynx by the movement of microvilli (19). The host then cough up the larvae which in turn is swallowed and transported to the small intestine. In contrast to *N.americanus, A.duodonale* can directly be swallowed from poorly rinsed vegetables. Stage L3 larvae develops into a worm that attach themselves to the duodenal mucosa (6), damages the intestinal mucosa and sucks 0,03-0,3 ml of blood per worm and day (15). After 5-8 weeks the worm reach sexual maturity and mate, resulting in the female worm releasing thousands of eggs each day into the intestinal lumen that are passed through the feces (3,15). Microscopic examination of stools can establish the diagnosis (19). Normally the hookworm lives inside its human host for several years (3,15).

Infection by hookworm are often asymptomatic though a consequence of decreasing hemoglobin levels is iron deficiency anemia (1,6). Anemia is a serious health condition that mainly affect women of a reproductive age and young children (7,20). Anemia and low iron count carries risks that include intrauterine growth retardation, low birth weight, preterm birth, maternal and infant mortality (7,9). Other consequences may include allergic reactions at the entry site, nausea, vomiting and diarrhea (15). The morbidity is directly related to the number of worms harboured (21). If the infection become chronical, emaciation as well as mental and physical retardation may occur because of the great loss of blood and other nutrients. Secondary infection by bacteria in the intestinal mucosa may also occur (15).

Diagnosis and treatment of hookworm infection

Because hookworm cannot be cultured, microscopic examination is the pillar of diagnostic parasitology. Other methods for parasitic diagnosis are available, for example molecular

analysis such as polymerase chain reaction (PCR) (3,15), that gives rapid, sensitive and specific diagnostic results. However, microscopy still remains the most common method of choice. This because of its low costs and simple techniques in combination with good results. The diagnosis of hookworm is established by first macroscopically examine the faecal samples for blood, mucus or other abnormalities (15). If dark stools are seen this indicate bleeding higher in gastrointestinal tract, while fresh blood indicate bleeding from the lower parts of the gastrointestinal tract. Presence of blood or mucus can come from a parasitic infection, but also from a number of different pathogenic conditions and should always be reported. Hookworm eggs are found in all types of faecal consistency; however, in very loose specimens' eggs may be hard to find because of the dilution factor (6). Hookworm eggs do not appear in the stool in consistent numbers on a daily basis, this because the eggs are intermittently released, therefore a number of three specimens should be collected at three separate days within ten days for an accurate diagnosis. It's recommended to collect in clean wide-mounted containers. Regularly a waxed cardboard or plastic container with a tight- fitting lid is used. The specimen should not be contaminated with water or urine because water contain free—living organisms that can be mistaken for human parasites and urine destroy motile organisms. In addition, urine also cause helminth eggs hatch (15). To prevent helminth eggs from developing to larvae and preserve the morphology of the eggs several fixatives can be used with the recommended ratio of stool and fixative. In order to ensure proper fixation 5% or 10 % buffered formalin, sodium acetate-acetic acid- formalin (SAF) or Schaudinns fluid polyvinyl alcohol can be used. The fixative kills any bacteria or viruses in the stools, which is an important safety aspect (6). The samples can be transported in a fixative SAF-tube and then directly analysed at the laboratory. In case the sample is transported unfixed the containers should be placed in plastic bags when transported to the laboratory and then fixed in a SAF-tube within 24 hours. In order to enable the microscopic analysis, the stools samples have to be prepared. This can be done with a variety of different methods including direct wet mount, Kato-Katz, concentration/sedimentation or flotation (6,22). Different methods are useful in detecting different range of parasites (15). The diagnosis can be based mainly on the presence of eggs, regardless of the amount found. In order to establish the intensity of an infection it is necessary to quantify the number, this also is important when treatment effects are studied (8).

Willis technique is a flotation method based on a solution containing saturated sodium chloride, with higher density than hookworm eggs. The gravity of hookworm eggs is 1.055 g/cm^3. The difference in density makes the helminth eggs float to the surface and adhere to a coverslip (23).

The sedimentation technique, also known as Formal ethyl acetate concentration method is based on a solution that have lower density than hookworm eggs, making the eggs sediment. Ethyl acetate is used to remove debris (6) and together with formalin fixed material it is the most common sedimentation method in routine laboratories (23). The sedimentation method is useful in diagnosis and recovering all types of helminth eggs.

Treatment of the symptoms caused by the infection include iron therapy, blood transfusions and nutrition complements. The symptom treatment does not decrease the transmission rate and does not cure the infection (24). In order to control the infection rate mass drug administration (MDA) with preventive chemotherapy are in use (5,14). Drugs such as a broad spectrum albendazole or mebendazole are common (15,24,25). Deworming drugs are often given as a single dose and most of the drugs available is considered to be cost-effective and side effects are relatively rare (1,14). However, studies show that the prevalence often returns after 6-18 month after treatment (25). As the deworming drugs does not prevent reinfection it's important to incorporate several strategies in order to evade new infection (15,24,25).

Risk factors and prevention

Contaminated water, inadequate sanitations supply's, crowded living and lack of knowledge increase the risk of infection. In regions where fertilizing with human feces is commonly used the prevalence of hookworm is significantly increased (1,5,14,26). Walking barefoot, not washing vegetables and hands, combined with unsafe disposal of faecal matter increase the risk of getting infected (14). Important steps include increased sanitary conditions, avoidance of contaminated soils and the use of enclosed shoes (15,19,26). Investigation of risk factors play a crucial role in the work regarding prevention of STH´s and providing educational material to those at risk (14). As the risk remains there is a need for the development of sufficient prevention strategies (20). There is an ongoing program in Vietnam with deworming that are used as one step to prevent anemia (9,20). It is of great importance that more studies take place in order to provide better understanding about the transmission spread (27).

Microscopic appearance of hookworm eggs

The eggs of the two species *A.duodenale* and *N.americanus* cannot be differentiated by basic light microscopy and is counted as hookworm combined. The eggs measure 55-75 µm x 36-40 µm and have a thin wall, is oval with rounded ends and contains an undeveloped embryo with 8-32 cells (22). A clean space between the embryo and shell is a distinct feature (19). A common similar specie, *Trichostrongylus spp* is often mistaken for hookworm eggs by unexperienced laboratory scientists. To separate the species, the size can be used as the *Trichostrongylus spp* is greater measuring 73-95 µm x 40-50 µm. *Trichostrongylus spp* also have thicker walls and are tapered at one end (6).

Purpose

The purpose of the field study was to identify the prevalence of hookworm infection in Da Nang, Vietnam using two methods, Willis flotation and Formal ethyl acetate concentration. Consecutive selected samples were used to evaluate any correlation between infection rate and individuals' gender, age and geographic area.

Materials and methods

Study population

The thesis is a quantitative, field study that took place in Vietnam at Da Nang University of Medical Technology and Pharmacy between 20th March to 15th of May 2016. A total of 101 participants were enrolled in the study. Totally 28 samples were consecutive obtained from individuals at Hoa Vang General Hospital, 18 samples from Son Tra Hosptial and 9 samples from Lien Chieu hospital. Further 14 samples were obtained from Hoa Vang district, 12 from Son Tra district, 4 Lien Chieu district and 16 from Ngu Hanh Son district Hoa Qug Ward. Samples from the community and the hospitals were collected in different ways and further combined according to geographic area. Samples from Lien chieu hospital and community, Son Tra hospital and community and Ngu Hanh Son represent the urban districts. The rural distrct is represented by samples from Hoa Vang hospital and community, see figure 1 for geographic location. The study population contained 46 men and 55 women that were further divided into different age groups, composed of 35 individuals in the age group 1-24, 44 in the age group 25-48 and 22 in the age group 49-72.

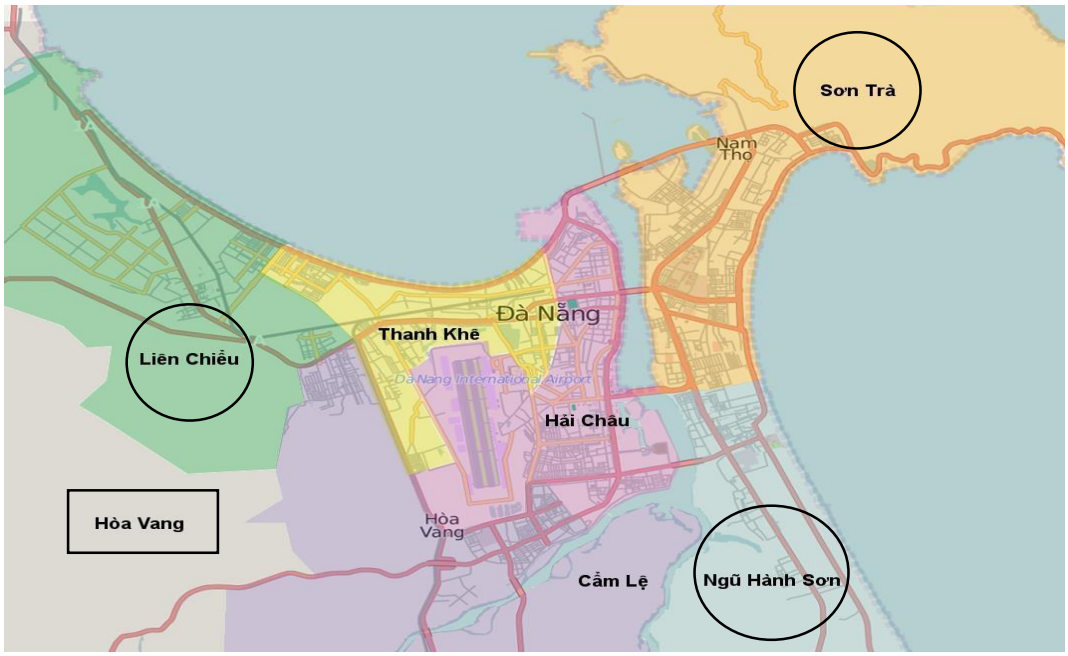

Figure 1; Map over Da Nangs districts, the urban districts Lien Chieu, Son Tra and Ngu Hanh Son, are marked with a circle. The rural district of Hoa Vang is marked with a square. (Map of Da Nangs districts. Downloaded 15th of May 2016 from; http://cvr.com.vn/en/blog/2015/05/02/choosing-the-right-place-to-live-in-da-nang/).

Stool sample collection and preparation

The participants were informed orally regarding the stool sample procedure, and given a clean wide-mounted container that then was submitted to a laboratory technician at respectively hospital mentioned above. All hospital based samples came from patients with symptoms from the gastrointestinal tract such as stomach pain and were requested by a doctor. The laboratory technician at the hospital who received the sample, removed a small part (4-5g) with the help of the accompanying spoon to a SAF-tube (1,5% formaldehyde solution, Microbiology department, Ryhov Jönköping). The tube was then transported to Da Nang University of Medical Technology and Pharmacy for analysis.

The community based samples were obtained by the staff at the different wards who asked patients to voluntary leave a stool sample. The individuals sought medical care for other reasons than of gastrointestinal tract issues. The voluntary individuals received a SAF-tube to take home where the collection took place, thereafter the SAF-tube was returned to respectably ward. The tube was then transported to Da Nang University of Medical Technology and Pharmacy for analysis. The SAF-tubes was pre-labelled with a code that follows the sample throughout the analysis and each patient were required to leave one sample each.

All of the collected samples were primarily macroscopically examined to ensure the collection tube contained 7ml SAF and one tablespoon of faeces (4-5gram). Further notes were taken regarding the consistency, colour and contents of the sample. The faeces sample was stored for at least 30 minutes in the SAF-tube before it was analysed. When the microscopic examination was performed notes were taken regarding the image quality as well as the number of eggs found in respectively method.

Preparation of solution was performed by a 0, 9 % sodium chloride (NaCl) (NaCl ≥99.5%. Sigma-Aldrich, Stockholm, Sweden) in distilled water respective a 1, 2 g/cm^3 saturated NaCl solution was mixed.

Two conical 15ml centrifuge tubes were marked with the same code as the SAF-tube, green coloured pencil for Formal ethyl acetate concentration and brown coloured pencil for Willis flotation. The fixated solution was mixed until a homogenous solution was achieved and the specimen was strained through a 7,5 x 7,5 cm wet gauze (AST Medical AB, Hisingsbacka,

Sweden) into a sterile cup. The solution was fully divided, 3 ml was added to respectively centrifuge tube with the help of a 3 ml Pasteur pipette (Passette, Alfa laboratorys limited Hampshire, UK), and further processed with Formal ethyl acetate concentration and Willis technique respectively.

Willis technique procedure

Saturated sodium chloride solution was added using a 3ml Pasteur pipette to the centrifuge tube until a meniscus was formed on the surface. The centrifuge tube was covered with a 21x26mm coverslip (Superior, W. Germany) for 10 minutes. A 76x26mm slide (Thermos scientific, Gerhald Menzel B.V Company, Braunschweig, Germany) was prepared during the time and then the coverslip was removed from the tube and placed on the slide for examination. The slide was placed in a Petri dish with a wet filter paper (Filter paper circles ø125mm, Whatman grade 589/1, Sigma Aldrich, Stockholm, Sweden) until analysed under a light microscope (LEICA DM 300, MicroMedic AB Stockholm, Sweden). First a 10X objective was used in combination with a 10X ocular. If a suspected hookworm egg was seen the objective was changed to 40X.

Formal ethyl acetate concentration technique procedure

A 3ml amount of solvent ethyl acetate, $CH_3COOC_2H_5$ (Xinlong Chemical CO., Ltd. Guangang Province China) were added using 3ml Pasteur pipette and the solution was shaken vigorously for one minute after a lid was putted on. After the centrifugation (Eppendorf centrifuge 5702, Eppendorf Nordic Aps, Horsholm, Denmark) at 1500 x g for three minutes, a sediment was obtained, three different layers consisting of formalin, plug of debris and ethyl acetate that was decanted to a slush. The pellet suspends in residual water and a 76 x 26mm slide were prepare for examination. A drop of the mixed pellet was placed on slides until all of the pellet was used. This was done with disposable pipette (Labdesign, Täby, Sweden) and one drop of 0,9% sodium chloride was added. Finally, the slide was mounted with a 21 x 26mm coverslip and then examined. The slide was placed in a Petri dish with a wet filter paper until it was analysed under a light microscope. First a 10 X objective was used in combination with a 10X ocular. If a suspected hookworm egg was seen the objective was changed to 40X.

Quality Control

To prevent false negative or false positive counts, the two authors examined the slides independent of each other and documented any individual differences in regard to the interpretation. In order to reduce the possibility of misdiagnosis of the hookworm eggs,

microscopic interpretation was also done by an experienced laboratory scientist. The samples obtained for Son Tra hospital, Lien Chieu hospital and Hoa Vang hospital had previously been diagnosed at respectively hospital by experienced laboratory technicians. The results from the hospital were checked against the study results.

Health care education

The ethical code of biomedical laboratory scientists involves the spread of knowledge, improvement and development of public health globally. Further responsibilities include development of existing and the establishment of new standards for optimal patient safety (28). In order to share preventive education about hookworm a poster with information regarding the spread of hookworm and how to decrease the risk of getting infection was given to Hoa Vang Hospital, see attachment 1. Hand desinfection gel was provided by Vårdförbundet Jönköping, Sweden and given to the student at university of Medical Technology and Pharmacy in order to increase the awareness of hygiene.

Ethical considerations

In agreement with Da Nang University of Medical Technology and Pharmacy the study needed no ethical review. All participants in the study were voluntary and leaving the survey was possible at any time. The sample collection procedure did not result in any physical discomfort or pain. Information regarding the study purpose was given orally in Vietnamese to all participants. The voluntary individuals that took part in the community based sample collection were informed regarding any positive results and were advised to seek medical attention. Further the samples were decoded to ensure anonymity and according to the Swedish Parliament law
2003:460 paragraph 3 an ethical trail is not necessary when participants can't be identified (29).

Statistical analysis

The prevalence of hookworm infection in relationship to age, gender and geographic area was calculated using the Chi-squared test Statistical analysis was completed using SPSS statistics software (version 19, 2012: Chicago, IL, USA).

Results

The macroscopic examination of the samples revealed no abnormalities such as blood, mucus or larvae. All collected specimens contained 7 ml of SAF and 4-5 grams of faeces.

Willis flotation and Ethyl acetate concentration

All 101 collected sample were analysed with Willis flotation technique and Formal ethyl acetate concentration. By both methods a total of 17 positive samples were found making the overall prevalence 16,8%. No significant quantitate differences between the two methods were establish and the independent analyse was interpret equivalent, the image quality was judged to be different between the both methods. In formal ethyl acetate concentration the image showed more debris in contrast to a cleaner image found in Willis flotation method, see figure 2a and 2b respectively.

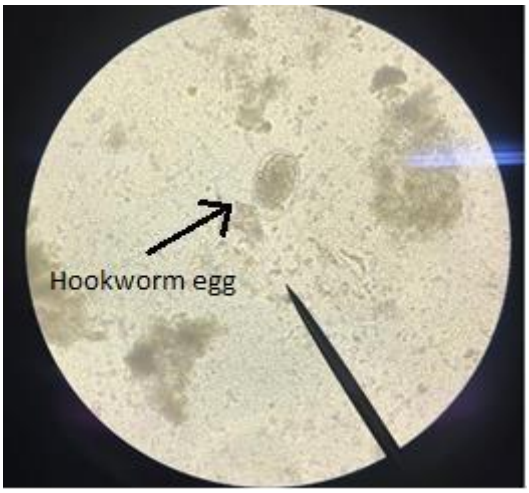

Figure 2a formal ethyl acetate concentration, image of hookworm egg, 400x magnification photo; Nyroz Agid, 2016-05-05

Figure 2b Willis flotation, microscopic microscopic image of hookworm egg, 400x magnification, photo; Nyroz Agid 2016-05-05

Gender

The registered 101 individuals were consisted of 46 men (46%) and 54 women (54%). Of the men 41/46 (89,1%) were negative, and 5/46 were positive for hookworm eggs making the percent in the male population study group 10,9%. The women in the study population revealed 43/54 (78,2%) negative, and 12/54 positives giving a 22, 2% infection rate among the female population. The total count in gender were 101, 84 (83,2%) negative, and 17 (16,8%) positive. The results of gender and hookwrom infection are shown in table I. There was no significant

difference according to gender (p=0,143) the prevalence of hookworm infection in the study population was thus found to be associated with gender.

Table I Prevalence of hookworm infection according to gender. The total negative and positive numbers of samples are shown, as well as the percentage within respectively gender. The total count and total percentage within both genders are presented. P-value 0,143

GENDER	Male	Count	41	5	46
		% within gender	89,1%	10,9%	100,0 %
	Female	Count	43	12	55
		% within gender	78,2 %	21,8%	100,0 %
TOTAL		Count	84	17	101
		% within gender	83,2 %	16,8 %	100,0 %

Age

Associations between age and hookworm infection revealed a higher infection rate within the age group 25-48 (p-value 0,035). The result is shown in table II.

Table II Prevalence of hookworm infection according to age. The total negative and positive numbers of samples are shown, as well as the percentage within each age group. The total count and total percentage within all the age groups are presented. P-value 0,035.

			NEGATIVE	**POSITIVE**	**TOTAL**
AGE	1-24	Count	33	2	35
		% within age	94,3 %	5,7 %	100,0 %
	25-48	Count	32	12	44
		% within age	72,7 %	27,3 %	100,0 %
	49-72	Count	19	3	22
		% within age	86,4 %	13,6 %	100,0 %
TOTAL		Count	84	17	101
		% within age	83,2 %	16,8 %	100,0 %

Prevalence of hookworm infection

Differences were assessed between geographic area and hookworm infection (p-value 0,001), were the rural district of Hoa Vang sowed 31,7% infection rate in contrast to 6,7% positive in the urban districts, see table III.

Table III Prevalence of hookworm infection according to geographic area divided into rural district and urban district. Number of negative, positive and total are shown as well as the percentage of the negative and positive results within the geographic area. The total number of positive and negative from both areas reveal the percentage of prevalence in the whole study area. P-value 0,001

			NEGATIVE	**POSITVE**	**TOTAL**
GEOGRAPHIC AREA	Da Nang Rural District (Hoa Vang Hosptial and community)	Count	28	13	41
		% within geographic area	68,3%	31,7%	100.0%
	Da Nang Urban District (Son Tra Hospital and community, Ngu Hanh Son Hoa Quy Ward, Lien Chieu Hospital and community)	Count	56	4	60
		% within geographic area	93,3%	6,7%	100,0%
TOTAL		Count	84	17	101
		% within geographic area	83,2%	16,8%	100,0%

Discussion

The prevalence of hookworm was evaluated in Da Nang Vietnam with two methods, the Willis flotation technique and Formal ethyl acetate concentration. By both methods hookworm eggs were visualized under light microscope and the quantitative differences were evaluated, as well as the image quality. At the sight of one or more eggs the samples were judged to be positive. In this study any realationship between infection rate and individuals' gender and age and geographic area, three urban districts (Son Tra, Ngu Hanh Son Hoa Quy ward and Lien Chieu), and one rural district (Hoa Vang) were assessed.

Methodological issues

Willis technique is a flotation method based on a solution containing saturated sodium chloride, with higher density than the eggs, making the helminth eggs float to the surface. The Formal ethyl acetate concentration method where ethyl acetate is used, is based on a solution that has lower density than the parasites, making the eggs sediment. An advantage of the flotation is that the image in the microscope is clear because of the low number of debris (23). Sedimentation usually leads to good results in the diagnosis, but it may be more difficult to detect the eggs in the microscope because of a very light weight debris. To reduce the amount of rubbish ethyl acetate attracts the debris and grease from faeces, making the microscopic image clearer (6). The Willis flotation use quite simple and cheap materials and can be used on unfixed material, whereas the formal ethyl concentration demands fixed samples as well as the chemical ethyl-acetate and a centrifuge, making it a more expensive method (18). The clearer image in the Willis flotation method resulted in quicker analysis in comparison with the Formal ethyl acetate concentration. A disadvantage is that Willis flotation can't detect very dense helminth egg since the high density makes them sink to the bottom. For example, *Ascaris spp.* eggs do not accumulate well using the flotation method (6).

A disadvantage of flotation is that the eggs might break and get filled with fluid giving it a higher density and descends to the bottom giving false negative results. The method should preferably be used on fixed material (23). An additional disadvantage that could have occurred is that the Willis flotation are more likely to miss light infections because of the fact that only one slide can be prepared. In contrast, the Formal ethyl acetate concentration gives rise to one or more slides as the pellet is larger giving more chances to find even a small amount of

hookworm eggs. In perspective of economics not all laboratories have the privilege to invest in a centrifuge or chemicals and simple flotation still provides good diagnostic tool if it is conducted correctly. For example, in developing countries such as Vietnam, the economics is of great importance and must be weight against the quality of the laboratory results. The laboratory work was judged to be trustworthy and the numbers of positive and negative samples found in the study to be truthful.

None of the 17 positive samples showed any significant quantitative difference between the two methods in detecting hookworm eggs in the study. As the purpose was to exclusively detect any presence of hookworm eggs, both methods proved to be sufficient. The experience regarding the microscopic image quality in the two methods was perceived differently. In the method Formal ethyl acetate concentration, the background image had more debris and was difficult to interpret while in the flotation method the image was clearer. However, ethyl acetate together with formalin fixed material is the most common sedimentation method in routine laboratories (22).

The remaining 87 samples were negative in both methods, false negative results could occur during the preanalysis, including the sample collection, transportation, storage and further during the laboratory process. Hookworm eggs do not appear in the stool in consistent numbers on a daily basis, this because the eggs are intermittently released. Therefore, the recommendation is to collect at least three samples every other day, within the maximum of ten days. If just one sample is examined the probability of detecting parasites is as low as 50-60%, but with the examination of three samples the likelihood of getting a positive result is >95% (22). The participants in this study were asked to leave one sample each, the reason was the time limitation, which may can have reduced the amount of positive results in the study. However, some literature suggests that in the case of only determine the presence or absent of eggs 1-2 samples are sufficient (15). It's important to gather the sample from both inside and outside of the feces in order to get a representative specimen. In order to get a correct fixation, the right SAF and feces ratio must exist (22), otherwise the eggs might hatch or lose its morphological characteristic. Control was conducted and a minimum of 7 ml SAF with 4-5g of feaces was decided, in all samples in the study, thus contained the correct amount. Another issue regarding fixation is if fresh specimen left in room temperature for more than 24 hours the eggs might hatch into larvae, giving false negative results (18).

Upon arrival to the laboratory no time had been noted on the SAF-tubes, nor did information exist on how long the sample had been standing at the hospitals before the fixation. In this study there was no control of how the preanalysis had been performed. With this in consideration it is possible that some positive samples may have been missed.

The samples in the SAF tube had to be mixed to a homogeneous solution and the solution was pored through the wet gaze. Some of the solution could get stuck in the SAF-tube or wet gaze, or insufficient mixing of the solution could result in low density infections being undetected. When the homogeneous solution was divided into respective centrifuge tube, the separation may have led to quantitative differences between the two methods. However, no such problems occurred during the laboratory work.

False negative result could occur if the solution with ethyl acetate is not vigorously shaken, leading to the microscopic image being unclear. If the mounted slide is left in room temperature or microscopic analysis is to slow the slide may dry giving rise to difficult interpretations. This was prevented by placing the prepared slides in a humid petri dish.

False negative result could occur when unexperienced biomedical technicians interpret the microscopic image. False positive results could also occur during the analysis because of misdiagnosis where *Trichostrongylus spp* are mistaken for hookworm eggs. Both the species are very similar in morphology (6). To prevent this the slides were analyzed independently by the two biomedical technician students. In order to reduce the possibility of misdiagnosis of the hookworm eggs, microscopic interpretation was also done by an experienced laboratory scientist, and the samples from the hospitals had previously been diagnosed. The results from the hospitals was crossed checked in order to reduce false results.

Result discussion

The consecutive selected samples were used to evaluate any correlation between hookworm infection and individuals' gender and age as well as geographic area.

Studies regarding the prevalence of hookworm in Vietnam are few, and those conducted are from the northern parts of the country. One study regarding women between the ages of 16-45

revealed a 76% infection rate (9). Additionally, a study from 2008, also conducted in the northern parts of the country, showed a prevalence of 52 % (10). In a study from 2013 conducted in the community of Dai loc district, Quang Nam province, located on the south coast of Vietnam, north of Da Nang, showed that 29% were infected by hookworm (12). In this study the prevalence was 16, 8 % which was lower than the previous studies conducted. The reason for this could be explanded by the big geographic distance between Da Nang and location of mentioned studies. The climate differs throughout the country (16) and suitable warmth, shade, optimal soil-type and humidity increase the risk of getting infected by hookworm. The rate of expansion for the hookworm larval and survival are dependent on the surrounding humidity and temperature. Further geographical differences such as rainfall and soil type may affect the transmission rate (10,14). The climate of Vietnam Da Nang have two season, a typhoon and wet season lasting from September through March and a dry season lasting from April through August. The temperature is typically high, with an annual average of 25,9 ^{o}C (78,6 ^{o}F) The annual average for humidity is 81% with a peak between October and December and lowest between June and July down to 60% and less. On average an 2,5 mm of rainfall is seen (16). During optimal conditions, like those found in Da Nang, with suitable warmth, humidity and shade the hookworm eggs hatch into infectious larvae within 24-48 hours (3). The climate in Da Nang seems to be optimal for hookworm and does not explain the lower prevalence in contrast to previous studies in the country.

Low income, low socioeconomically status and living in agricultural areas are known to increase the risk of getting an infection by STH´s (14). In contrast Vietnam has undergone massive development during the past ten years and most people have access to running water and a higher sanitation standard (30). Additional reasons could be the prevention of hookworm infection from the national program of preventing helminths as demand of government that focus on wide range drug for children and providing education for the public (14,31). Maybe the result differs between the provinces throughout the country because of many things, such as large/small area/population, custom of living and how the medical staff work. The relationship between high risk for hookworm infection and occupation has been investigated in different studies. In Sichuan Province, China and in Vietnam, high infections rates were found and highly associated with the widespread use of faeces as night-soil fertilizer. Among families who work with agricultural hookworm has also been shown to be more common (3).

Studies have been conducted to evaluate if there could be any gender-difference in hookworm infection. For example, in South China and Vietnam the females have a higher infection ratio. In a study from Vietnam the explanation could have been that the women were responsible for the night-soil use. The difference regarding hookworm infections according to gender is most likely explained by gender-related exposure rather than susceptibility difference between the sexes (3). In northern Vietnam, a study found that women had an infection rate at 67% and men 60%, the difference could be explained due to the fact that women spent most time working in the rice fields (10). In the results from this study no differences between the sexes was found. The number of 101 participants probably had an impact on the result, where a larger number of individuals would have increased the chances of getting p-value demonstrating differences. Future studies should take place, containing more individuals, in order to establish any relationships in regards to hookworm infection and gender in the area.

The results are consistent with other research in this matter, studies from China and Southeast Asia indicate a prevalence peak in the middle age, or even the age of 60 for hookworm infections (3). Two other studies give support to this statement (10, 11). The age group consist of people in the middle age, making them the best suited for working in the fields and in the forest. The children and old are probably not working in the farms, giving them a decreased risk of getting infected.

The three urban districts showed 6,7% positive samples, a significant lower infection rate than the rural district, where 31,7% samples were positive. Da Nang have three urban district; Lien Chieu, Son Tra and Ngu Hanh Son, Hoa Quy Ward with people manly working in tourism service and are less involved in farming. The rural district, Hoa Vang has a large area, where a small part is used as an industrial zone while the large part is used for agriculture and forest planting. The results in this study are consistent with previous researches, where higher infection rates were found in rural districts (11,18,27). The income and educational rate in the areas may help explain the difference in hookworm infection (16). Partly the risk factors are permeant, working in soil were the eggs live under optimal conditions. Perhaps not wearing shoes, because of economic reasons, also increase the risk. Distance to local health care centres and limitations in public transportation makes people isolated, eventually hindering them from reaching the deworming programs.

Conclusion

With the help of the two methods Willis flotation technique and formal ethyl acetate concentration the presence of hookworm eggs in fixed faecal samples were identified under a light microscope. A total of 101 individuals from Da Nang City, Vietnam participated in the study and the result showed a 16,8% infection rate. Correlation between gender, age and geographical area against infection were investigated. No association could be found between hookworm infection and the participants' gender. In the age group 25-48 a higher infection rate was found in contrast to other age groups. Further correlation was established between infection rate and geographical area, where the rural district of Hoa Vang showed a higher infection rate compared to the urban districts.

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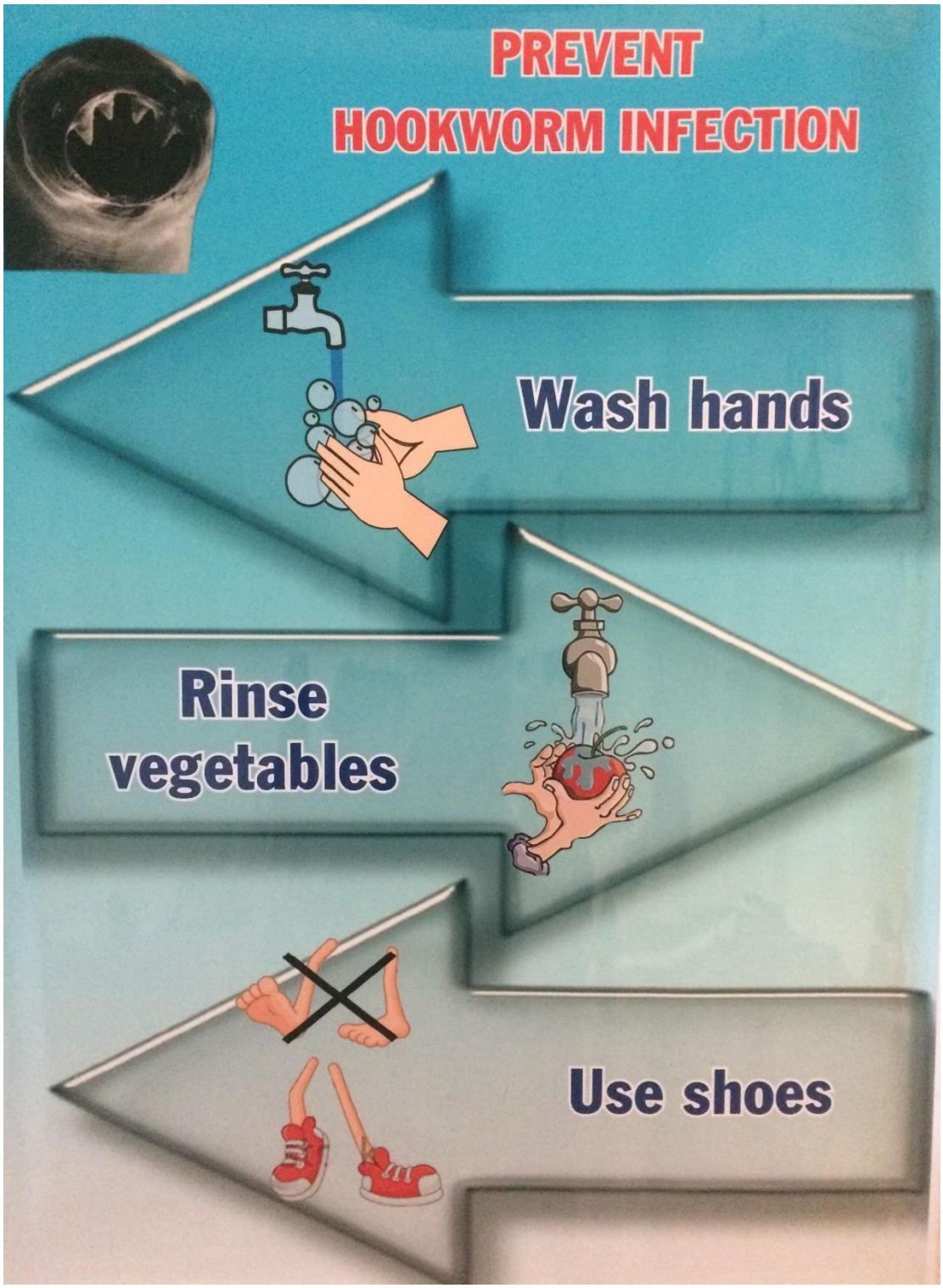
Attachment


PREVENT
HOOKWORM INFECTION
Wash hands
Rinse
vegetables
Use shoes