Diagnosis and epidemiology of *Strongyloides stercoralis* in Cambodia

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Dekan

Dedicated to my beloved family

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List of abbreviations

ABZ	Albendazole
AIDS	Acquired immunodeficiency syndrome
ATB	Atabrine
BCI	Bayesian confidence interval
BM	Baermann
bp	base pair
CAP	*Carica papaya*
CNM	National Center for Parasitology, Entomology and Malaria Control
Cox-1	Cytochrome c oxidase 1
Ct	Cycle threshold
DNA	Deoxyribonucleic acid
EKBB	Ethikkommission beider Basel
ELISA	Enzyme-linked immunosorbent assay
EPG	Eggs per gram
ERR	Egg reduction rate
FBZ	Flubendazole
FECT	Formalin-ether concentration technique
GDP	Gross domestic product
GEV	Gentian Violet
HIV	Human immunodeficiency virus
HTLV-1	Human T-lymphotropic virus 1
HVR	Hyper variable region
IgE/G/M	Immunoglobulin E/G/M
IL	Interleukin
IPI	Intestinal parasitic infection
ITS	Internal transcribed spacer
IVM	Ivermectin
KAP	Koga Agar plate culture
KK	Kato Katz
LAMP PCR	Loop-mediated isothermal amplification PCR

x

Lao PDR	Lao People's Democratic Republic
LPG	Larvae excreted per gram stool
LRR	Larvae reduction rate
MBZ	Mebendazole
MZR	Mirazid
NECHR	National Ethics Committee for Health Research
NTD	Neglected tropical disease
od	oral dose
OR	Odds ratio
PAP	Pyrantel Paomate
PCR	Polymerase chain reaction
PRZ	Piperazine
PVP	Pyrvinium Paomate
RNA	Ribonucleic acid
rpm	rounds per minute
SAF	Sodium-acetat formaldehyde
SEA	Southeast Asia
SSU	Small ribosomal sub-unit
STH	Soil-transmitted helminth
Swiss TPH	Swiss Tropical and Public Health Institute
TBM	Tribendimidine
TBZ	Thiabendazole
TH1/TH2	T-helper cell 1/2
TPS	Trichlorophenol-Piperazine Salt
USD	US Dollar
WHO	World Health Organisation

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Summary

Background: The soil-transmitted helminth *Strongyloides stercoralis* is the most neglected of all neglected helminth infections. It is known to occur worldwide, more prominently in tropical and subtropical resource-poor areas. Environmental and socio-economic factors such as inadequate sanitation contribute to high infection numbers. Today's estimates for the number of people infected with *S. stercoralis* globally are cited to be around 30-100 million people, however based on insufficient studies. Compared to the three widely known STHs, namely *Ascaris lumbricoides*, hookworm and *Trichuris trichiura*, in most countries, still only fragmentary information is available about the prevalence of *S. stercoralis*. A main factor contributing to this knowledge gap is the lack of high sensitivity diagnostic methods. The most commonly applied diagnostic methods for helminths have a very low sensitivity for detection of *S. stercoralis*. There are more appropriate diagnostic methods, yet they are not routinely applied. Furthermore these methods rely on multiple sampling for sensitivity increase, mainly because the irregularities with which *S. stercoralis* larvae are excreted in low-intensity infections. With Ivermectin, a safe and highly efficacious drug is available for the treatment of *S. stercoralis*, yet it is not routinely used in helminth control programs.

Cambodia, located in Southeast Asia, provides ideal conditions not only for STH-infections, but also other helminthic and protozoan infections. However, there is still only little data available on *S. stercoralis*. In the rural communities, where people mainly rely on subsistence farming, there is a constant exposure to risk factors for STH-infection.

Furthermore, specific details about the parasite, its biology and genetics, as well as transmission dynamics remain poorly understood. E.g., excreted larvae are detected in diagnostic tests, however, excretion dynamics are poorly investigated. Another aspect important for control is the possible zoonotic potential of *S. stercoralis*. *Strongyloides* infects animals and humans. If and how frequent infection between animals and humans occurs is unknown.

Aims and objectives: The work presented in this PhD was divided into three sections. The first section of the work consisted in examining today's literature to draw a more accurate picture of the global prevalence, diagnosis and risk factors for *S. stercoralis*. Furthermore we aimed at analysing information about the treatments available for *S. stercoralis* today. The second section focused on *S. stercoralis* in communities in an endemic setting. We aimed of assessing co-infections, risk factors and transmission dynamics. The third section focused on the individual level of infection with *S. stercoralis*. We aimed to assess the excretion pattern in individuals before treatment and the impact of treatment, we genotyped larvae collected

from humans and animals, to get more insight into possible transmission between humans and animals, and we validated a new PCR assay for the detection of *S. stercoralis*.

Methods: For the first section a literature search was conducted including all available studies reporting on the prevalence of *S. stercoralis* in communities and from hospitals. The data was combined and used for a meta-analysis to give prevalence estimates per country. Further were possible risk factors analysed with the help of a meta-analysis. A second review was conducted collecting information on the treatment of strongyloidiasis. Pooled cure rates were established for all drugs included in a meta-analysis.

The second and third section of this work were based on field work conducted in Kandal, Preah Vihear and Takeo province in Cambodia. To assess multiparasitism in humans and animals, a community-based survey was conducted. Inhabitants of rural villages were enrolled, and stool samples collected. The samples were analysed with several diagnostic methods: Kato Katz, Baermann, Koga Agar plate culture, formalin-ethyl acetate concentration method and PCR. Animals samples were analysed with the flotation method and PCR.

The studies reported in the third section were undertaken in the framework of our large-scale community-based surveys in Preah Vihear and Takeo province. For the study on the excretion pattern of *S. stercoralis* larvae, we used a modified Baermann method. By weighing the amount stool used, we established the larvae per gram stool that were excreted. For the genotyping of single worms, the worms were collected either from the Baermann or Koga Agar plate culture and fixed in 70% ethanol for subsequent molecular analysis. For the collection of *S. stercoralis* from animals, a veterinarian was appointed. This enabled us to collect the stool directly from the animals.

Results and conclusions: In total, 354 studies were included in the *S. stercoralis* prevalence review. Almost two thirds (63.3%) of these studies applied low sensitivity diagnostic methods. Serology was only used in 9.9% of the studies. Only two countries report 40 or more studies, namely Brazil (43 studies) and Thailand (40 studies). For many countries, potentially endemic for *S. stercoralis*, no data is available, e.g. in sub-saharan Africa where we could identify studies only in 20 (43.5%) of the 46 countries.

We conclude that the prevalence of *S. stercoralis* is still underreported. We demonstrated that there are still many gaps and "black spots" on the world map remaining today. Diagnostic methods most commonly applied in studies on STH infections today are not appropriate for the detection of *S. stercoralis*, which is one of the main factors for the underreporting of the prevalence.

We included 68 studies for the review on treatment. Efficacies of 14 different drugs were reported. The four drugs tested the most include Ivermectin (in 30 studies), Albendazole (in 23 studies), Thiabendazole (in 21 studies) and Mebendazole (in 5 studies). A meta-analysis revealed the best pooled cure rate for Ivermectin with 0.97 (95% BCI: 0.94 – 1.00) and Thiabendazole with 0.95 (95% BCI: 0.92 – 1.00).

We conclude that Ivermectin is the best available treatment. Our studies further confirmed the effectiveness and safety in application of ivermectin. As ivermectin already has been applied in mass drug administration (MDA) programmes, for instance in the treatment of lymphatic filariasis, inclusion of ivermectin in helminth control MDA-programmes is an option to be considered in endemic areas. Other potential candidates we report for treatment of *S. stercoralis* need to be further evaluated, as the heterogeneity of available data (diagnostic methods applied, reports of adverse events) makes it difficult to assess and compare the established cure rates.

Our field survey conducted in rural villages in Preah Vihear province, Cambodia, revealed that intestinal multiparasitism was very frequent, in humans as in animals. From 218 humans examined, 191 (87.2%) had at least one intestinal parasitic infection. We could document 14 different parasitic species; eight helminthic and six protozoan parasites. In humans, most frequently diagnosed were hookworms (63.3%), *Entamoeba* spp. (27.1%), *S. stercoralis* (24.3%), *Giardia duodenalis* (22.0%) and *Blastocystis hominis* (18.4%).

In the 94 dogs sampled, the most common infections diagnosed were hookworm (80.8%), *Spirometra* spp. (21.3%) and *Strongyloides* spp. (14.9%). Altogether, we detected eleven parasitic species (eight helminhts and three protozoans). Seventy-seven (81.9%) had at least one infection diagnosed. In 76 pigs, 74 (97.4%) had at least one infection diagnosed. Of the twelve detected parasitic infection (eight helminths and four protozoan), the most frequent were *Isospora suis* (75.0%), *Oesophagostomum* spp. (73.7%) and *Entamoeba* spp. (31.6%).

The molecular analysis of hookworms revealed that 50.8% of the hookworm infected humans are due to *A. ceylanicum*. In the dogs positive for hookworm, 90.0% were infected with *A ceylanicum*.

In conclusion, we could demonstrate the high prevalence of intestinal multiparasitism in rural villages in Cambodia, not only in humans but also in domestic animals. We documented that infection with *A. ceylanicum* is rampant in humans in rural villages, and we showed the potential for zoonotic transmission between dogs and humans. We argue that for prevention and control, integrated approaches are needed, including not only the humans but the animals as well.

Our studies focusing on the individual level of strongyloidiasis infection, we analysed the excretion pattern of *S. stercoralis* larvae. Thirty-nine positive cases were followed for seven days prior to ivermectin treatment. Larvae per gram stool (LPG) were calculated. The highest and lowest average density per person observed were 151.2 LPG (range: 28.8 – 410.6 LPG) and 0.03 LPG (range 0 – 0.07 LPG), respectively. Local maxima estimation technique did not detect a pattern of excretion, indicating that larvae excretion is random and does not follow a cyclic pattern. High-intensity infections (>10 LPG every day) were correctly diagnosed *S. stercoralis* positive every day. In low-intensity infections, patients did not excrete larvae for up to two consecutive days. They would have been wrongly diagnosed as infection-free in a one day examination.

Those results underline that low-intensity infections can easily be missed if only single stool samples are analysed, as the individuals can have excretion-free days. This further emphasizes the importance of analysing samples of the same individual on consecutive days to increase sensitivity of the diagnostic methods and to subsequently better estimate the true prevalence

We evaluated the performance of real-time monoplex PCR for detection of *S. stercoralis*. We compared the PCR assay with the results of coprological diagnostic methods (Baermann and Koga Agar plate culture). We estimated an overall sensitivity and specificity of 61.0% and 92.7%, respectively. The cycle-treshold (ct) values indicate that PCR most often failed to detect infection in low-intensity infections. We conclude that it is a suitable option for individual diagnosis, but is difficult to apply in field settings.

We performed a molecular analysis of single *S. stercoralis* worms. We sequenced 269 individual worms collected from 29 individuals. We could identify three different genotypes of *Strongyloides* in our study population, co-existing sympatrically within the same host. Yet, we were not able to detect heterozygous worms, which would indicate that there is no interbreeding.

With our large-scale studies conducted in Cambodia, we could demonstrate the presence of high infection rates of *S. stercoralis*, never been reported at that high level in Cambodia. It shows that applying good sensitivity diagnostic methods on consecutive days gives prevalence rates that are a closer to the true prevalence and considerably higher than what has been previously reported. We conclude that this is true for most *S. stercoralis* endemic settings and hence, the true prevalence is likely to be higher than the numbers reported in studies which apply low sensitivity diagnostic methods.

1. Introduction

The purpose of this work is to give an overview about today's knowledge about the prevalence, diagnostic possibilities and the treatment of the soil-transmitted helminth (STH) *Strongyloides stercoralis*. It further wants to put more light into some specific aspects of the biology and genetics of *S. stercoralis*. Aspects of co-infection and risk factors are analysed in an endemic setting, bridging the gap and showing the challenges for the control, not only in Cambodia, but in a worldwide picture.

1.1 Strongyloides stercoralis

1.1.1 Classification and history

S. stercoralis is a parasitic nematode and belongs to the genus *Strongyloides*. Grove summarized 52 different *Strongyloides* species, all obligate gastrointestinal parasites of vertebrates exist (Grove 1989). There are two species of *Strongyloides* known to infect humans: *S. stercoralis* and *S. fuelleborni*. While *S. stercoralis* is known to occur worldwide, the distribution of *S. fuelleborni* is restricted. It is mainly found in African primates, which can share the infection with humans (Hira and Patel 1980; Pampiglione and Ricciardi 1972). A subspecies, *S. fuelleborni kellyi,* is only found in humans in Papua New Guinea (Ashford et al. 1992).

Louis Normand was the first person to detect and describe *S. stercoralis* in 1876. He analysed stool samples of French soldiers returning from Chochin-China (today Vietnam) that were complaining mainly about diarrhea. The life-cycle, clinical features and pathology were only fully disclosed in the 1930ies.

1.1.2 Life cycle

S. stercoralis is transmitted by the penetration of the intact skin by infective filariform larvae (L_3) (Figure 1.1 – Nr. 1). The larvae are attracted by different chemicals which a suitable host releases. One main attractant is thought to be uronacic acid (Safer et al. 2007). After the larvae entered a host, they migrate via the blood system to the lungs, break out of the capillaries and reach the alveoli. Moving up the respiratory tree, they arrive in the pharynx and are eventually swallowed. By this they reach the intestines. The larvae feed on the intestinal mucosa of the duodenum and upper jejunum of the small intestine (2). They mold twice to become adult female worms (3). There are no male adult worms in the intestine. The adult female worms live in the mucosal epithelium. They reproduce parthogenetically and produce numerous eggs per day (4). The eggs hatch in the gut and rhabditiform larvae (L_1) emerge. The newly hatched rhabditiform larvae (5) can undergo two different developments:

1. Direct or homogonic development: the rhabditiform larvae (L_1) (1) mold twice to become infective filariform larvae (L_3). They are released via the stool of the host. Once they are in the environment, they can infect a new host by penetrating the intact skin (1). Additionally, compared to other nematodes, *S. stercoralis* has the unique feature for the so-called auto-infection. In this case, the newly developed infective filariform larvae (L_3) infect the same host by penetrating the colonic wall or the perianal skin (6). With the auto-infection, they can initiate a new parasitic cycle in the same host. This feature enables *S. stercoralis* to maintain long-lasting infections in the same host.

2. Indirect or heterogonic development: rhabditiform larvae (L_1) leave the host with the faeces and then develop via a total of four stages into adult male and female worms (7). These free living worms can then reproduce sexually (8,9). The offspring can develop either into infective filariform larvae (L_3) and infect a new host (1). They can also sustain the free-living cycle if they develop again into male and female adult worms (10).
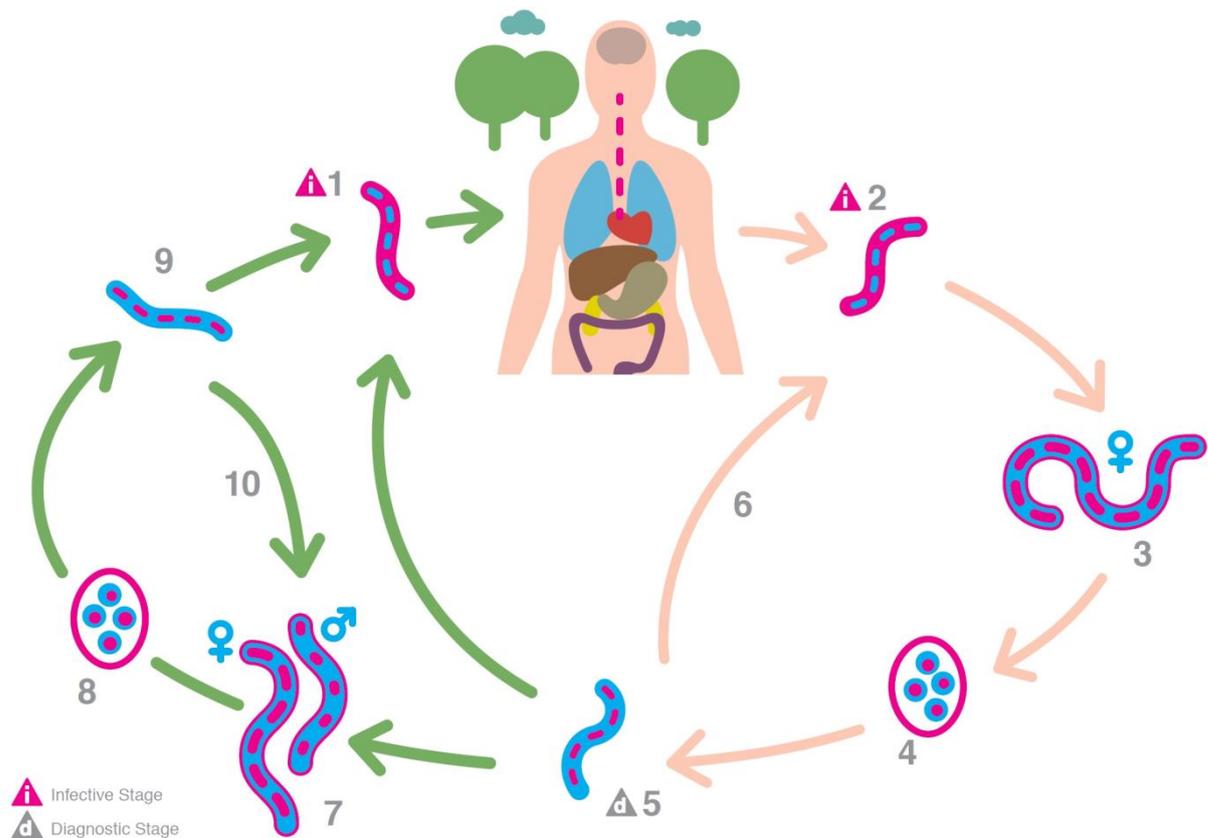
Figure 1.1 – The life-cycle of *S. stercoralis* (adapted from CDC)

1.1.3 Autoinfection

The possibility of infecting the same host again is a feature unique to *S. stercoralis*. It is a major problem for the treatment of *S. stercoralis*. Unlike with other helminthic infections, it is necessary to eradicate all larvae and adult worms in an infected individual. Otherwise one single adult can reproduce again and re-infect the "treated" individual via auto-infection. The individual therefore remains in fact still infected with *S. stercoralis*. There are several reports of sustained auto-infections with *S. stercoralis*, sometimes for up to 75 years (Concha et al. 2005; Genta 1992; Keiser and Nutman 2004; Prendki et al. 2011; Vadlamudi et al. 2006).

1.2 Morphology and biology of *S. stercoralis*

1.2.1 Adult stages

In infected hosts, only female adult worms exist. These parasitic females are 2 to 2.8 mm long and between 35-40 µm in width (Figure 1.2). They live in the intestinal mucosa and reproduce parthogenetically. The eggs are located in the uterine area, and all other organs are pressed against the body wall. The eggs are released through the vaginal opening. The free living rhabditiform females are generally smaller (1 to 1.5 mm length) but wider (85 µm). In the free living form also males exist. They are smaller than the free-living females with a length of 0.95 to 1.2 mm and a width of 55 µm. In the free-living cycle, there is a sexual reproduction. The males inseminate the females with their copulatory spicules.

Figure 1.2 – Morphology of *S. stercoralis*. a) Mating of male and female adult *S. stercoralis*, b) Almost fully embryonated egg of *S. stercoralis*, c) Female adults and larvae.

1.2.2 Larvae

The third-stage filariform larvae (L$_3$) are about 400-700 µm in length and 12-20 µm in width, meaning they are comparably long and thin. The rhabditiform larvae (L$_1$-L$_3$) are 250 µm in length as L$_1$ and continually grow to become L$_3$-larvae. The larvae of *S. stercoralis* resemble larvae of hookworm (*N. americanus* and *A. duodenale*). For diagnosis, they can be distinguished as follows: rhabditiform larvae of *S. stercoralis* have a characteristic short buccal cavity and a prominent genital primordium. The filariform larvae of *S. stercoralis* are characterized by a long oesophagus, making up almost 1/3 of the body length of the larvae.

1.2.3 Eggs

The eggs of *S. stercoralis* are thin-shelled and ellipsoidal. They are about twice as long as wide and measure 40-70 µm in length. They resemble the eggs of hookworm. Yet, they are rarely seen, as the parasitic female lays the eggs in the intestinal epithelium, where they hatch before being released with the stool (Figure 1.2).

1.3 Genetic aspects of *S. stercoralis*

The genotyping of *S. stercoralis* is focused on the small ribosomal sub-unit (SSU) rDNA sequence, which is highly conserved. It therefore is ideal for phylogenetic studies among and within species (Blaxter et al. 1998; Floyd et al. 2002; Hasegawa et al. 2009; Herrmann et al. 2006). The SSU sequences are seen as useful markers for molecular taxonomy in the genus *Strongyloides* (Dorris et al. 2002; Eberhardt et al. 2008; Hasegawa et al. 2009; Hasegawa et al. 2010). To know genetic variations helps to establish more insight into possible transmission routes, especially between humans and animals. Yet, until today, only a few studies on within-species variations in the SSU and in mitochondrial DNA sequences have been conducted, all on *S. stercoralis* originating from different locations and hosts (Hasegawa et al. 2009; Hasegawa et al. 2010; Hu et al. 2003; Koosha et al. 2009; Pakdee et al. 2012). Studies of the genetic structure of *S. stercoralis* poplations within a specific location are missing altogether.

1.4 Clinical outcome and pathology

It has been estimated that around 50% of all infections with *S. stercoralis* remain as asymptomatic, chronic infections (Concha et al. 2005). There is a wide range of mild or moderate symptoms associated to the infection with *S. stercoralis* (Figure 1.3). They most often relate to the skin or the gastro-intestinal tract. Larva currens is a condition in which the migrating larvae lead to a serpiginous, itchy rash. The rash stems from an allergic reaction to

the migrating larvae. Symptoms of the gastro-intestinal tract can include diarrhea, weight loss, abdominal pain and/or discomfort and pruritus ani (Grove 1996; Liu and Weller 1993; Mahmoud 1996).

If the immune system of the host is compromised, there is a range of more severe symptoms that can occur. The impairment of the immune system, either due to underlying disease or therapeutic interventions (especially with corticosteroids) can result in an increase of auto-infective larvae. If these larvae migrate through the body according to their migratory route it is called the hyperinfection syndrome. The major complications that arise include pulmonary symptoms like fever, productive cough, shortness of breath, pleural effusion, pulmonary abscess and can ultimately lead to respiratory failure. Gastro-intestinal symptoms include massive diarrhea, protein-losing enteropathy, hypoalbuninaemia and generalized oedema, intestinal pseudo-obstruction, ulcerative colitis, bowel infarction, lower gastro-intestinal bleeding and biliary obstruction. Neurological symptoms include headache, nausea, vomiting, focal neurological signs, bacterial meningitis and brain abscess (Grove 1996).

If the larvae spread out to other tissues and organs, this state is called disseminated strongyloidiasis. The symptoms occurring in disseminated strongyloidiasis depend on the organs involved, including the small and large intestines, stomach and oesophagus, hepato-biliary system, pancreas, respiratory tract, cardio-vascular system, central nervous system, urinary tract and reproductive system and the skin. Both conditions, the disseminated strongyloidiasis and the hyperinfection syndrome show a high mortality if untreated (Siddiqui and Berk 2001).
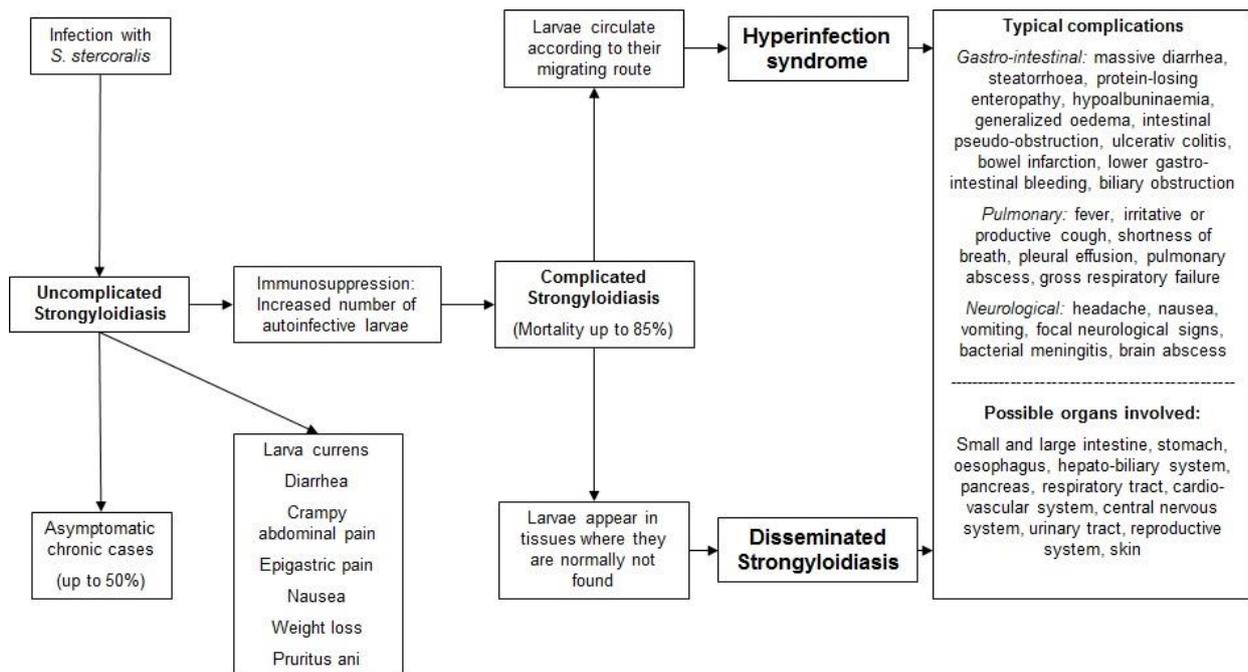
Figure 1.3 – Symptoms associated with *S. stercoralis*.

1.5 Risk factors: HTLV-1, HIV and alcoholism

Besides the environmental and socio-economic risk factors for infection with *S. stercoralis*, other risk factors for infection have been described. Persons infected with the human T-lymphotropic virus 1 (HTLV-1), a retrovirus, showed a higher risk of being co-infected with *S. stercoralis* (Chieffi et al. 2000; Courouble et al. 2004; Einsiedel and Woodman 2010; Nera et al. 1989). The HTLV-1 targets mainly T-cells and alters the immune response of the infected individual. In persons co-infected with HTLV-1 and *S. stercoralis*, there is a high production of Interferon gamma, which decreases the production of several molecules (e.g. IL-4, IL-5, IL-13 and IgE) involved in the immune response against helminthes, increasing the risk for disseminated strongyloidiasis. Furthermore is the efficacy of treatment for *S. stercoralis* reduced (Carvalho and Da Fonseca Porto 2004).

The acquired immunodeficiency syndrome (AIDS) can occur in individuals infected with the Human Immunodeficiency Virus (HIV). Strongyloidiasis has originally been included in the list of AIDS-defining diseases, yet today, it no longer constitutes an AIDS-defining, opportunistic infection (WHO 2007) as it did during the onset of the HIV-pandemic. Today, most case-control studies comparing HIV-infected individuals and non-infected individuals report an increased risk for *S. stercoralis* infection, but only in a few studies this difference is significant (Feitosa et al. 2001; Gomez Morales et al. 1995; Pinlaor et al. 2005). There are also reports, where the *S. stercoralis*-prevalence is similar or even lower in HIV-infected individuals (Dias et al. 1992; Fontanet et al. 2000; Lebbad et al. 2001; Mendez et al. 1994). Not many cases are reported in which strongyloidiasis would cause severe complications like hyperinfection in HIV-positive individuals (Keiser and Nutman 2004). Today it is thought that modulation of the immune system by the HI-Virus appears to be the main reason for this, as it increases TH2 cytokines and decreases TH1 cytokines (Barker et al. 1995; Tanaka et al. 1999; Valdez and Lederman 1997). This modulation leads to an immune response pattern that may favour bacterial and viral opportunistic infections rather than helminthic infections (Concha et al. 2005). Another reason is thought to be that the indirect larval development is promoted in patients that are immuno-compromised by advancing AIDS and therefore, the possibility of increased auto-infection is reduced (Viney et al. 2004).

Alcohol-addiction is another potential risk factor for *S. stercoralis* infection. There only a few studies, undertaken in Brazil (de Oliveira et al. 2002; Marques et al. 2010; Zago-Gomes et al. 2002). They all showed an increased risk for *S. stercoralis*-infection with high ethanol intake. In the study by Zago-Gomes and colleagues (Zago-Gomes et al. 2002) the higher risk was shown to occur for *S. stercoralis* infection, but not for other nematode infections. They argue that alcoholics' regular ethanol intake might lead to an immune modulation and/or alteration in corticosteroid metabolism, favoring *S. stercoralis* infection.

1.6 Diagnosis

1.6.1 Coprological diagnostic methods

In most surveys focusing on STHs, there is one coprological diagnostic method applied, namely the Kato Katz (KK) method. It has a high sensitivity for the detection of most helminth eggs present in the faeces. Yet for *S. stercoralis*, where the larvae and not the eggs are excreted by the host, this is not the case. Until today, there is no gold-standard diagnostic method for the detection of *S. stercoralis*. From the coprological diagnostic methods available, the Koga Agar plate culture (KAP) and the Baermann method (BM) are the methods with the highest sensitivities and considered to be the most appropriate.

The KAP method is a cultivation method, in which stool is placed on a freshly produced Agar plate. It is incubated at 28ºC for two days. During this time, the larvae of *S. stercoralis* move out of the stool into the growth medium. This can be shown by the tracks left by moving larvae, as they are covered in bacteria, which start to grow in the paths left behind (Figure 5). Using the KAP method, it is also possible to observe adult *S. stercoralis*. For the diagnosis, the cultured Agar plates are washed out with sodium-acetat-formaldehyd (SAF) solution. After centrifugation, the sediment can be read under a light microscope. Additionally, the KAP method is used for diagnosis of hookworm. If eggs are present in the stool analysed, they will hatch during the two days of incubation. It is therefore a crucial point to distinguish between larvae of *S. stercoralis* and hookworm larvae, especially if co-infections exist. This is achieved by differentiating the larvae by morphological features. The rhabditiform larvae of *S. stercoralis* can be distinguished by their characteristic short buccal cavity and the prominent genital primordium; the filariform larvae is characterized by a long oesophagus.

The BM method is easier to apply than the KAP if material and time are considered. On the other hand, it needs larger amounts of stool (around 25-30g vs. 3-5g in the KAP). For the diagnosis, the stool is placed in a funnel that is equipped with a gauze and a wire-mesh. The funnel is filled with tap water. A clamp prevents the water from flowing out. The whole installation will be left for two hours with artificial light placed at the bottom of the funnel. Larvae will move out of the stool and fall through the mesh and gauze and are collected at the bottom of the tube. After removal of the clamp, the water is collected in a tube and subsequently centrifuged. After centrifugation, the sediment can be read for larvae present (Figure 1.4).
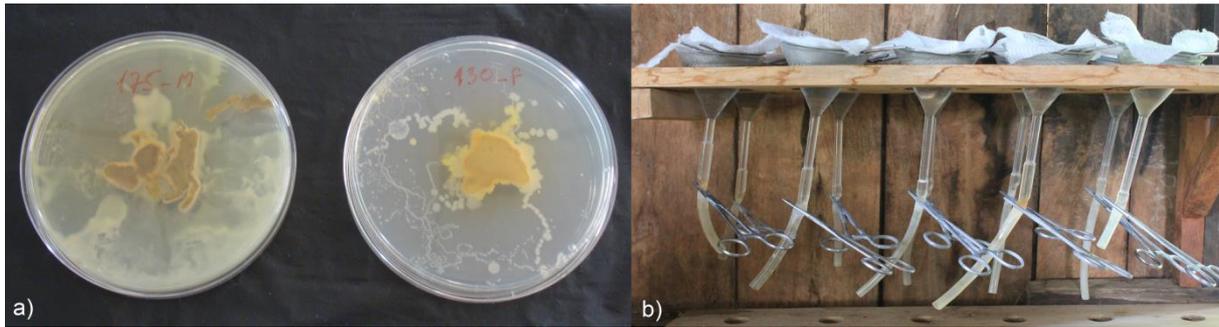
Figure 1.4 – Diagnostic methods for *S. stercoralis*. a) Koga Agar plates, negative (left) and positive (right) for *S. stercoralis,* b) Baermann set up.

For both methods it is important to consider that laboratory staff is working with infective material. In the BM, the larvae collected and centrifuged are still living and infective. Great care must be taken to prevent infection. In the KAP, it is important to carefully handle the Agar plates, as they can contain infective larvae as well.

1.6.2 Serological diagnostic methods

There are serological methods available for the detection of *S. stercoralis* such as the Enzyme-linked Immunosorbent Assay (ELISA). The ELISA has a high sensitivity, yet serological antibody tests can have cross-reactivity if other helminth infections are present (Gam et al. 1987; Grove 1996; Lindo et al. 1996). For assessing the sensitivity it is a problem that there is no gold standard diagnostic method available for *S. stercoralis*. Furthermore, the ELISA is hard to use in remote field settings, as sophisticated laboratory material is required. The ELISA works is detecting the serum IgG against a crude extract of the filariform larvae of *S. stercoralis*. Many studies evaluating the ELISA are undertaken in proven cases of Strongyloides after parasitological detection of larvae in stool samples. A major constraint is that demonstration of antibodies does not distinguish between current or past infections, and the time period necessary for sero-conversion is unknown. Animal trials showed the IgM turn positive one week after experimental infection of dogs with *S. stercoralis* and IgG showed a rise soon after (Grove and Northern 1982). But equivalent studies in humans are not available and therefore the utility of immunoassays for both diagnosis and follow-up after treatment remain somewhat controversial (Loutfy et al. 2002).

1.6.3 Molecular diagnostic methods

The polymerase chain reaction (PCR) can be used for detection of *S. stercoralis* infection. This molecular diagnostic method uses primers that are species-specific to amplify DNA or

RNA that might be present in a sample analysed. The primers have to be constructed to target parts of the genome that are exclusive for the target. In the case of *S. stercoralis*, the primers consist of *S. stercoralis*-specific sequences like the cytochrome c oxidase subunit I gene and the 18S rRNA gene sequence (Verweij et al. 2009). To test the sensitivity of the *S. stercoralis*-PCR, several studies used symptomatic or already diagnosed cases. In these samples, the PCR showed high sensitivities of up to 100% (Basuni et al. 2011; Janwan et al. 2011; Moghaddassani et al. 2011; Taniuchi et al. 2011; ten Hove et al. 2009; Verweij et al. 2009; Zeehaida et al. 2011). Studies on asymptomatic cases, i.e. in a field setting are scarce, and have yet to prove the usefulness of PCR diagnosis. An additional challenge is the sophisticated laboratory equipment needed for performing a PCR analysis and the comparably high costs. Today, there are also so-called multiplex PCRs available which are able to detect several intestinal parasites (including *S. stercoralis*) (Taniuchi et al. 2011). Generally, the PCR shows high potential for the diagnosis of *S. stercoralis*, yet it is more feasible to apply in well-established laboratories. For the screening of suspected cases (i.e. in returning travellers) it might offer an alternative diagnostic possibility.

1.7 Treatment

Compared to the treatment of other STHs, the treatment for strongyloidiasis should target the total eradication of the parasite from the host (Grove 1996; Liu and Weller 1993). This is because *S. stercoralis* has the ability to induce autoinfection in the same host. If the worm burden is only reduced, but the parasite not completely eradicated, the host can be re-infected and a new reproduction cycle starts, sustaining the infection despite treatment. Today, there are two drugs available with a known high cure rate, namely Ivermectin and Thiabendazole. Thiabendazole is known to cause a variety of side-effects, ranging from general fatigue, dizziness, headache, nausea, appetite loss, abdominal pain up to liver dysfunction and neuropsychiatric symptoms (Grove 1982). Ivermectin is the therefore considered the preferred treatment (Adenusi 2003; Behnke et al. 1994; Caumes et al. 1993; Datry et al. 1994; Gann et al. 1994; Heukelbach et al. 2004a; Heukelbach et al. 2004b; Igual-Adell et al. 2004; Lindo et al. 1996; Marti et al. 1996; Naquira et al. 1989; Ordonez and Angulo 2004; Shikiya et al. 1991; Shikiya et al. 1994; Toma et al. 2000; Zaha et al. 2000; Zaha et al. 2002). Mebendazole and Albendazole, both widely used for the treatment of other STH infections, effect *S. stercoralis* as well, although showing lower efficacy compared to Ivermectin and Thiabendazole. Both of these dugs therefore should not be recommended, especially as a single dose, because they are likely to fail to eradicate the parasite from the host (Krolewiecki et al. 2013).

Because of the challenges of diagnosing infection with *S. stercoralis*, especially the low-intensity chronic infections, it is difficult to assess the true cure rate for the different treatments.

1.8 *S. stercoralis* in animals

Animals, including domestic animals can harbour many different helminthic and protozoan infections and can act as a host. Dogs, for instance, are associated with more than 60 zoonotic parasites worldwide, many of which pose serious public health concerns (Eguia-Aguilar et al. 2005). In rural communities, people rely on and live in close proximity with their domestic animals.

Figure 1.5 – Domestic animals in Cambodia. a) & b) Pigs and dogs living in close contact with their owners.

In Cambodia, the most important domestic animals include cows, pigs, dogs, chicken and water buffaloes. The water buffaloes are mainly kept for helping in the field work (i.e. for ploughing the rice fields). The dogs are mainly guard dogs at night, but roam free in the villages at day time. There are also a considerable number of stray dogs, as the animals are not sterilized. All other animals are kept for the own food consumption or the sell in markets. The close proximity of which humans and animals live together coupled with poor levels of sanitation, hygiene and a lack of veterinary attention could pose a possible risk factor for transmission between humans and animals or vice versa. This is especially true for pigs and dogs, as these two animals are mainly kept on the household premises (Figure 1.5). In both animals, several intestinal parasites have been documented to have a zoonotic potential. Namely in dogs, *Strongyloides* spp. (Viney and Lok 2007), hookworm (Areekul 1979),

T. canis (Colli et al. 2010), *Echinostoma* spp. (Lan-Anh et al. 2009), *G duodenalis* (Traub et al. 2004a) and *Entamoeba* spp. (Dado et al. 2012). In pigs, *Ascaris* spp. (Anderson 1995), *Trichuris* spp. (Nissen et al. 2012), *B. coli* (Owen 2005), *Capillaria* spp. (Fuehrer et al. 2011) and *Entamoeba* spp. (Verweij et al. 2001). In the case of *S. stercoralis*, it is known that there are different genetic strains in animals and humans, and that human strains can infect animals (Eberhardt et al. 2008). Yet, even if *S. stercoralis* can infect humans and animals alike, the transmission between them has not been documented.

1.9 *S. stercoralis* and other soil-transmitted helminths

Besides *S. stercoralis*, there are three major soil-transmitted helminths (STHs) known to exist world-wide, namely *Ascaris lumbricoides*, the hookworms (*Necator americanus* and *Ancylostoma duodenale*) and *Trichuris trichiura* (Bethony et al. 2006). It is estimated that 819 million people are infected with *A. lumbricoides*, 465 million with *T. trichiura* and 440 million with hookworm (Pullan et al. 2014). All three belong to the family of nematodes, similar to *S. stercoralis.* Infection with STHs either occurs when the eggs are ingested (*A. lumbricoides* and *T. trichiura*) or by penetration of the hosts skin by infective larvae (hookworm and *S. stercoralis*). The eggs/larvae survive best in humid or moist conditions in tropical or subtropical areas. Especially the eggs of *A. lumbricoides* can survive for a long time because of their thick shell (Maya et al. 2012). Many countries in the developing world have ideal conditions for the survival of STHs and additionally the sanitary conditions and the occupation of the inhabitants (especially farmers) are contributing to the high infection rates found. Access to sanitary facilities has been shown to have a protective effect for infection with all three major STHs (Ziegelbauer et al. 2012). Major risk factors for infection with *A. lumbricoides* and *T. trichiura* include the use of night soil for fertilization. Eggs are distributed in the field with the contaminated faeces used for the fertilization of the crops. The eggs survive on the crops and with unhygienic food handling/preparation of these crops can lead to the eggs being ingested by a suitable host. For hookworm and *S. stercoralis* not wearing shoes, especially combined by occupational exposure like working as a farmer, is a major risk factor for infection, as the larvae can penetrate the intact skin of a suitable host. The larvae are distributed with the faeces. They survive best in humid, wet soil (hence: soil-transmitted helminths). If a suitable host gets in contact with the faecally polluted soil, the larvae can penetrate the skin and infect the host. Most of these risk factors, whether ecological or economical can be found in many tropical and subtropical countries in the developing world, which is underlined by the fact that the highest infection rates are found in said countries (Figure 1.6).

Behavioural	Environmental
• Toilet usage • Personal cleanliness • Occupation (e.g. farmer) • Wearing shoes	• Tropical climate • High humidity • Unhygienic sanitation • Land surface temperature • Night soil fertilizer

Figure 1.6 – Factors influencing the transmission of STHs (adapted from Ohta et al, 2007)

Figure 1.7 – Rural Cambodia. a) Sanitary facilities, b) Typical housing in rural villages, c.) Risk factors: children walking barefoot

In South-east Asia (SEA), STHs have been demonstrated to be highly endemic (Brooker et al. 2003). In Cambodia, Lao PDR, Thailand and Vietnam, there are ideal ecological

conditions for the survival and transmission of STHs existing (Figure 1.6). In most parts of these countries there is a tropical, humid and warm climate all year round. Furthermore are the socio-economic conditions in most of the rural areas in these countries contributing to high transmission rates. In Cambodia, there are often no or very poor sanitary facilities in the rural areas. Most of the residents rely on subsistence farming, with rice growing being the most important occupation. During the work in the field, farmers get exposed to several risk factors for infection with STHs. In most of the surveys focusing on STHs, *S. stercoralis* is neglected. The following chapters shed light into the reasons why this is the case.

2. Aim and objectives

2.1 Aim

The aim of this work is to give an overview about today's knowledge about the prevalence, diagnostic possibilities and the treatment of *S. stercoralis*. It further wants to put more light into some specific aspects of the biology and genetics of *S. stercoralis*. Aspects of co-infection and risk factors are analysed in an endemic setting, bridging the gap and showing the challenges for the control, not only in Cambodia, but in a worldwide picture.

2.2 Main objectives

- To assess todays knowledge about the global prevalence of and the risk factors for infection with *S. stercoralis*.

  → Review the literature and summarize prevalence data.

  → Establish prevalence estimates on a country basis.

  → Summarise and analyse information on the risk factors for *S. stercoralis.*

- To compare the existing diagnostic methods and treatment regimens for *S. stercoralis*.

  → Review the literature on data about the diagnostic methods available for *S. stercoralis.*

  → Evaluate and compare the different treatments available for *S. stercoralis.*

- To document the dynamics of co-infections in a *S. stercoralis* endemic setting and to assess genetic variability of *S. stercoralis* and the potential of human-animal transmission.

  → Collect and analyse stool samples from humans, dogs and pigs living in rural households in Cambodia.

  → Molecular analysis of hookworm infection.

- Conduct detailed studies on molecular aspects of *S. stercoralis* and the treatment thereof at individual level.

  → To assess the excretion of larvae in infected individuals.

  → Document the effect of Ivermectin treatment on the excretion of larvae.

  → Evaluate real-time PCR for the detection of *S. stercoralis.*

  → Genotype individual *S. stercoralis* worms from humans and animals.

3. Material and methods

3.1 Approach

To complete the aim and the objectives of this thesis, the work was divided into three sections. The first section consisted of a literature review on *S. stercoralis*, including the prevalence, diagnosis, risk factors and treatment. The second section focused on *S. stercoralis* at the household level, while the third section focused on the individual level of *S. stercoralis* infection. The second and third sections were both achieved with field work conducted in Cambodia.

3.2 Cambodia

Cambodia is a country located in South-East Asia. It shares borders with Thailand, Lao PDR and Vietnam. In 2008, 13.4 Million inhabitants lived in Cambodia; the estimate for 2010 is more than 14.9 Mio inhabitants. More than 1.3 Mio people live in the capital Phnom Penh. The climate in Cambodia is tropical, with a wet rainy season altering with a dry season, with temperatures averaging 30ºC all year round. The country remains one of the poorest countries in South-East Asia, with an estimated 2400 USD GDP per capita in 2012. More than 50% of the inhabitants have to live with less than 2 USD per day. In the rural parts of the country, the main occupation is subsistence farming. In our study, there were two provinces selected for conducting the field work. The main focus lied on Preah Vihear province, which is one of the poorest and most remote provinces in Cambodia. It covers an area of 13'788 km^2. In 2008, 171'000 people lived in Preah Vihear province, averaging in 12 persons per km^2. Almost 94% of inhabitants live in rural areas (National Census, Cambodia, 2010).

3.3 Field sites and surveys conducted

For this work, data was collected during 5 individual field surveys in Cambodia (Figure 3.1).

2009: A cross-sectional study was conducted in four schools in Kandal province, south of Phnom Penh. Children were screened using the Baermann method, Koga Agar plate culture, Kato Katz method, FECT, providing three stool samples each.

2010: A community-based cross-sectional survey was conducted in Preah Vihear province. Sixty villages have been randomly selected, and around 15 households per village were enrolled. More than 3000 persons have been sampled with Baermann, Koga Agar, Kato Katz and FECT. Additionally samples were stored for PCR analysis. Stool samples were provided

on two consecutive days. A sub-sample of the participants was enrolled for an excretion study.

2011: A community-based cross-sectional survey was conducted in Takeo province. Again 60 villages were randomly selected, and around 15 households each enrolled. All samples were analysed with the Baermann, Koga Agar, Kato Katz and FECT. One sample per person was analysed. Another sub-sample was enrolled for an excretion study.

2012: Another community-based cross-sectional survey was conducted in the same villages with the same individuals enrolled in 2010 in Preah Vihear province to determine incidence of re-infection and effect of treatment. All samples were analysed with the Baermann, Koga Agar, Kato Katz and FECT. One sample per person was analysed.

2013: A second follow-up survey was conducted again in the same villages in Preah Vihear province. Additionally, we visited additional villages for the collection of larvae from humans and animals.
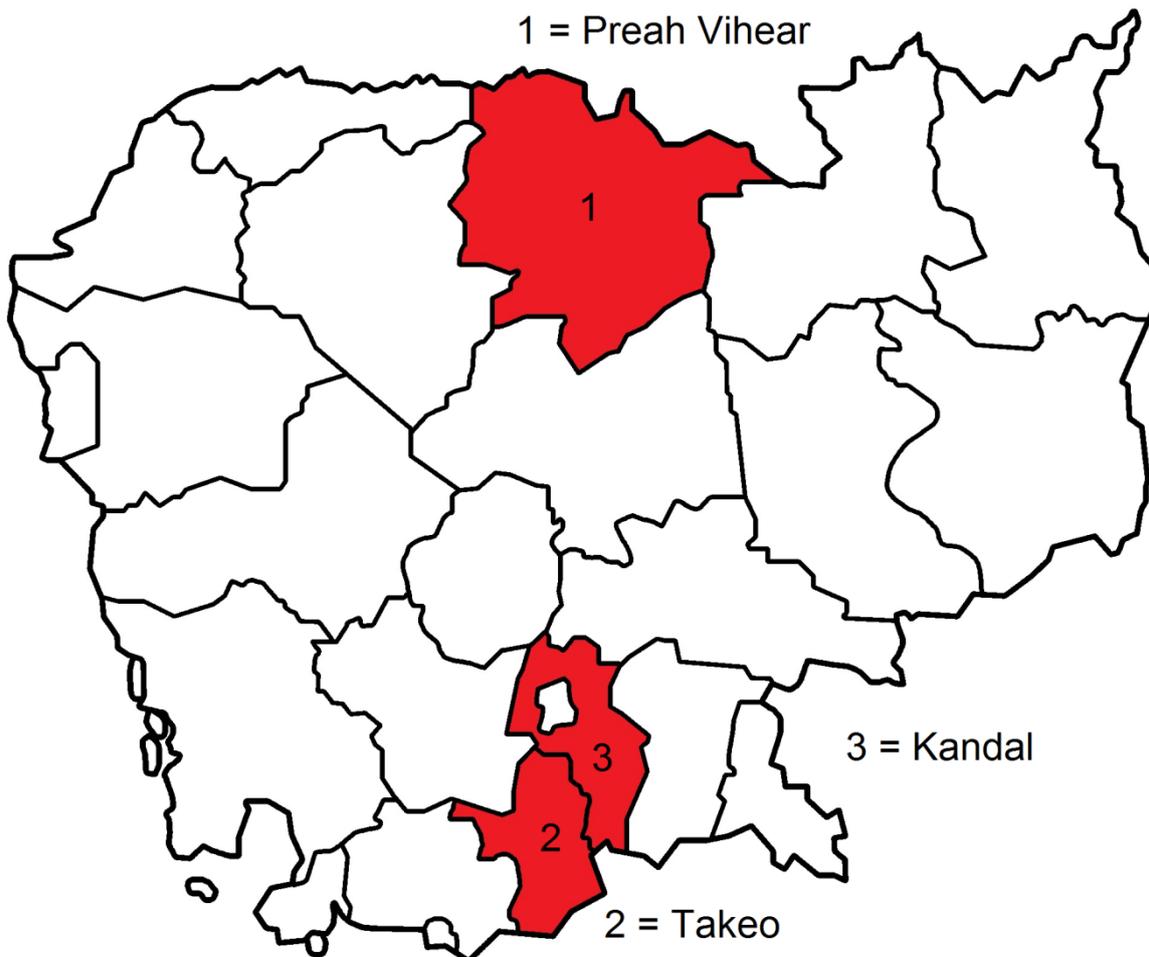
Figure 3.1 – Map of Cambodia, with Preah Vihear, Takeo andKandal province indicated

3.4 Ethical considerations

All study protocols for the surveys undertaken were approved by the Ethics Committee of the Cantons of Basel-Stadt and Baselland, Switzerland and the National Ethics Committee for Health Research, Ministry of Health in Cambodia. All participants and relevant parties were informed of the purpose of the study. Written informed consent was obtained from all individuals prior to enrolment. All infections with *S. stercoralis* were treated with Ivermectin, 200μg/kg bodyweight. All other infections diagnosed were treated according to the national Cambodian treatment guidelines.

4. Outline of thesis

The PhD-Thesis is divided into three main parts, each with its own chapters, followed by a general discussion (Chapter 12):

1. **Prevalence, treatment and risk factors for *S. stercoralis***
   a) Chapter 5: *Strongyloides stercoralis*: Global prevalence and risk factors.
   b) Chapter 6: Treatment of *Strongyloides stercoralis.*

2. **Cambodia: *S. stercoralis* and co-infections**
   c) Chapter 7: The prevalence and diversity of intestinal parasitic infections in humans and domestic animals in a rural Cambodian village.
   d) Chapter 8: High prevalence of *Ancylostoma ceylanicum* hookworm infections in humans, Cambodia, 2012.

3. **Specific aspects of *S. stercoralis:* Improving diagnosis and uncovering transmission dynamics**
   e) Chapter 9: Evaluation of real-time PCR for *Strongyloides stercoralis* and hookworm as diagnostic tool in asymptomatic schoolchildren in Cambodia.
   f) Chapter 10: *Strongyloides stercoralis* larvae excretion pattern before and after treatment.
   g) Chapter 11: *Strongyloides stercoralis* genotypes in humans in Cambodia

5. *Strongyloides stercoralis*: Global Distribution and Risk Factors

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5.1 Abstract

5.1.1 Background

The soil-transmitted threadworm, *Strongyloides stercoralis*, is one of the most neglected among the so-called neglected tropical diseases (NTDs). We reviewed studies of the last 20 years on *S. stercoralis's* global prevalence in general populations and risk groups.

5.1.2 Methods/Principal Findings

A literature search was performed in PubMed for articles published between January 1989 and October 2011. Articles presenting information on infection prevalence were included. A Bayesian meta-analysis was carried out to obtain country-specific prevalence estimates and to compare disease odds ratios in different risk groups taking into account the sensitivities of the diagnostic methods applied. A total of 354 studies from 78 countries were included for the prevalence calculations, 194 (62.4%) were community-based studies, 121 (34.2%) were hospital-based studies and 39 (11.0%) were studies on refugees and immigrants. World maps with country data are provided. In numerous African, Asian and South-American resource-poor countries information on *S. stercoralis* is lacking. The meta-analysis showed an association between HIV-infection / alcoholism and *S. stercoralis* infection (OR: 2.17 BCI: 1.18 – 4.01; OR: 6.69; BCI: 1.47 – 33.8), respectively.

5.1.3 Conclusions

Our findings show high infection prevalence rates in the general population in selected countries and geographical regions. *S. stercoralis* infection is prominent in several risk groups. Adequate information on the prevalence is still lacking from many countries. However, current information underscore that *S. stercoralis* must not be neglected. Further assessments in socio-economic and ecological settings are needed and integration into global helminth control is warranted.

5.2 Introduction

The threadworm *Strongyloides stercoralis* is a soil-transmitted nematode and one of the most overlooked helminth among the neglected tropical diseases (NTDs) (Olsen et al. 2009). It occurs almost world-wide, excluding only the far north and south, yet estimates about its prevalence are often little more than educated guesses (Bethony et al. 2006; Genta 1989). Compared to other major soil-transmitted helminths (STHs), namely *Ascaris lumbricoides* (roundworm), *Necator americanus* and *Ancylostoma duodenale (*hookworms) and *Trichuris trichiura* (whipworm), information on *S. stercoralis* is scarce (Bethony et al. 2006). The diagnostic methods most commonly used for STH detection, such as direct fecal smear or Kato-Katz, have low sensitivity for *S. stercoralis* or fail to detect it altogether (Sato et al. 1995a; Siddiqui and Berk 2001; Steinmann et al. 2007). Especially the parasitological diagnostic tools for *S. stercoralis* infection like the Koga Agar plate culture consume more resources and time than the most commonly applied methods (Agrawal et al. 2009) and hence, are rarely used in potentially endemic settings of resource poor countries.

S. stercoralis was first described in 1876. The full life cycle, pathology and clinical features in humans were fully disclosed in the 1930s (Figure 5.1). The rhabditiform larvae are excreted in the stool of infected individuals. The larvae mold twice and then develop into infective 3rd stage filariform larvae (L$_3$), which can infect a new host by penetrating intact skin. The larvae thrive in warm, moist/wet soil. Walking barefoot and engaging in work involving skin contact with soil, as well as low sanitary standards are risk factors for infection. Hence, many resource poor tropical and subtropical settings provide ideal conditions for transmission (Concha et al. 2005; Genta 1992; Viney 2006).

S. stercoralis is an exception among helminthic parasites in that it can reproduce within a human host (endogenous autoinfection), which may result in long-lasting infection. Some studies report individuals with infections sustained for more than 75 years (Concha et al. 2005; Genta 1992; Keiser and Nutman 2004; Prendki et al. 2011; Vadlamudi et al. 2006). Two other species, closely related to *S. stercoralis*, also infect humans, namely *S. fulleborni* and *S. cf fulleborni*, which are of minor importance and geographically restricted (Ashford et al. 1992; Dorris et al. 2002).

S. stercoralis' ability to cause systemic infection is another exceptional feature of the threadworm. Particularly in immunosuppressed individuals with a defective cell-mediated immunity, spread from the intestinal tract of one or more larval stages may lead to hyperinfection syndrome and disseminated strongyloidiasis, in which several organs may be involved (Grove 1996). The outcome is often fatal (Fardet et al. 2006; Marcos et al. 2008;

Siddiqui and Berk 2001). In contrast, uncomplicated intestinal strongyloidiasis may include a spectrum of unspecific gastro-intestinal symptoms such as diarrhea, abdominal pain and urticaria (Grove 1996; Khieu et al. 2013b).
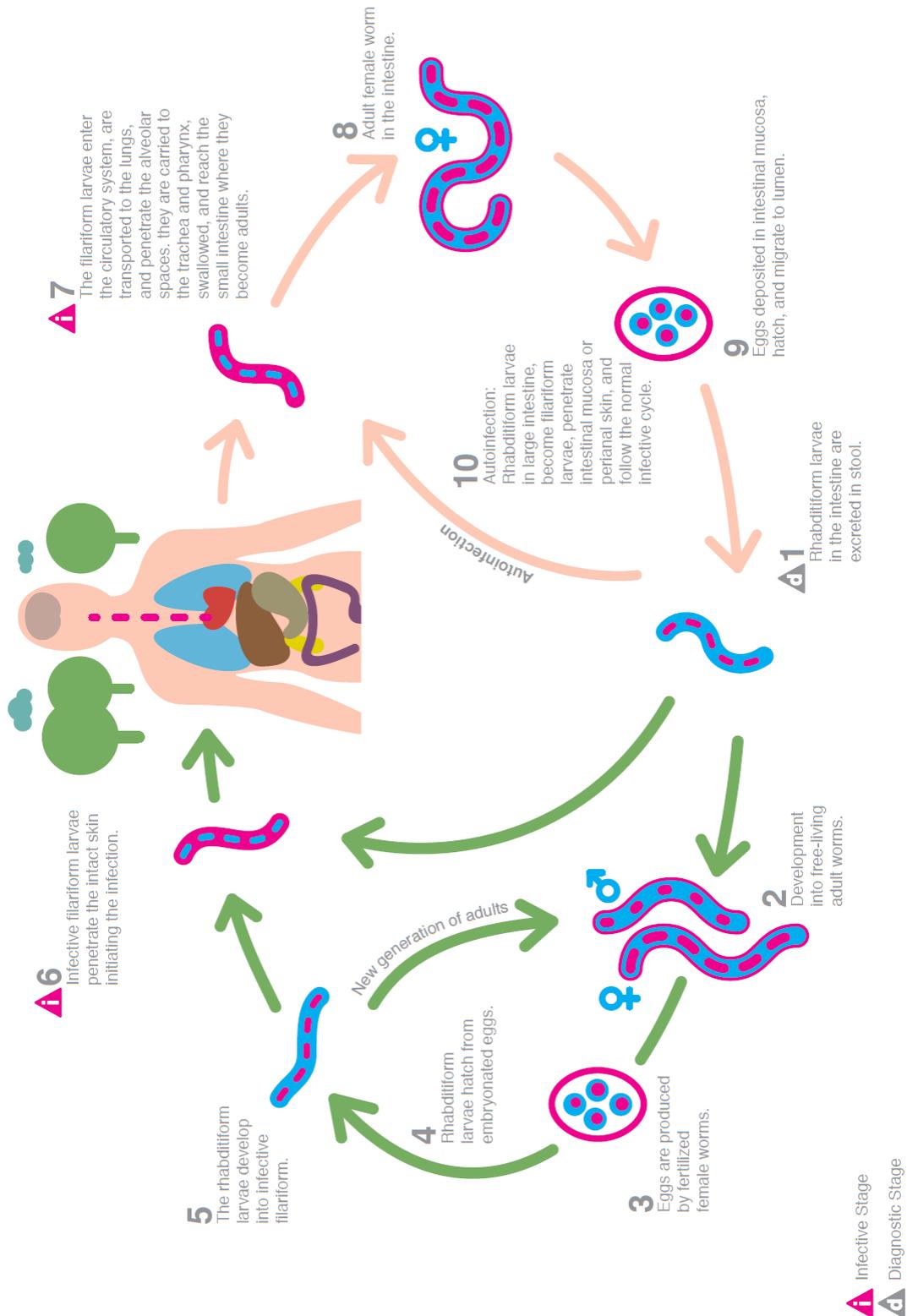
Figure 5.1 – The life-cycle of *S. stercoralis*

However, most infections, chronic low-intensity infections in particular, remain asymptomatic. Asymptomatic infections are particularly dangerous. In cases of immunosuppressive treatment, especially with corticosteroids, they have the potential to develop fatal disseminated forms. Proper screening of potentially infected individuals before immunosuppressive treatment (coprologically over several days and/or serologically) is essential, though often not carried out. This asymptomatic infection, coupled with diagnostic difficulties, (often due to irregular excretion of parasite larvae) leads to under-diagnosis of the threadworm. Assessing the clinical consequences of infection remains challenging, thus, little is known about the *S. stercoralis* burden in endemic countries.

In 1989, Genta (Genta 1989) summarized information on global distribution of this parasite for the first time. He found *S. stercoralis* to be highly prevalent in Latin America and sub-Saharan Africa. He further pointed out that many reports suggested high infection rates in South-East Asia and described several risk groups, including refugees and immigrants.

The objectives of our study are to obtain country-wide estimates of *S. stercoralis* infection risk in the general population, and to assess the association between *S. stercoralis* prevalence and different risk groups. We reviewed the available literature and carried out a Bayesian meta-analysis taking into account the sensitivity of the different diagnostic tools. The models allowed estimation of the diagnostic sensitivity for different study types and risk groups.

5.3 Materials and Methods

5.3.1 Literature search and data extraction

We conducted a systematic literature review of all research papers published between January 1989 and October 2011 and listed in PubMed. Papers were filtered using the search terms "*Strongyloides*" or "*Strongyloides stercoralis*" or "Strongyloidiasis". Studies were included if they contained information on prevalence and/or risk of *S. stercoralis* infection, either in the general population or in risk groups, i.e. patients with HIV/AIDS, immuno-deficiencies, HTLV-1-infection, alcoholism, and diarrhea.

We excluded articles (i) that were not written in English, Spanish, Portuguese, French or German language; (ii) that referred to specific bio-molecular research aspects of *S. stercoralis*; (iii) on infection in animals, and (iv) that did not provide additional information on the prevalence and/or risk of *S. stercoralis* infection.

For each selected paper, the following information was recorded: number of infected individuals, number of examined individuals, risk factors (specific risk group or control group), study area (country or geographic coordinates, when available) and WHO world region (Region of the Americas, European region, African region, Eastern Mediterranean region, South East Asia region and the Western Pacific region), study type (cross-sectional, case-control etc.), place of implementation (community- or hospital-based studies, and studies on refugees and immigrants), and diagnostic procedures used (copro-diagnostic, serological methods etc.).

5.3.2 Statistical analysis

The main outcome of the analysis is *S. stercoralis* prevalence in the general population for each country as well as in specific risk groups, namely HIV/AIDS patients, HTLV-1 patients, alcoholics and patients with diarrhea.

A Bayesian model for meta-analysis that included the diagnostic-test sensitivity was formulated and implemented in WinBUGS 1.4 (Lunn et al. 2000).

Information about the sensitivity of the different diagnostic tools used was derived from the literature and led to the division of diagnostic procedures into three sensitivity groups. We assigned a range of sensitivity using the lowest and the highest sensitivity reported, respectively (Arakaki et al. 1990; de Kaminsky 1993; Gyorkos et al. 1990; Hernandez-Chavarria and Avendano 2001; Huaman et al. 2003; Koga et al. 1990; Koga et al. 1991; Koosha et al. 2004; Lindo et al. 1994; Mahdi et al. 1993; Mahmoud 1996; Mangali et al. 1991; Marchi Blatt and Cantos 2003; Sato et al. 1995b; Sato et al. 1990; Schaffel et al. 2001; Sithithaworn et al. 2003; Speare and Durrheim 2004; Sudarshi et al. 2003; Sukhavat et al. 1994; Uparanukraw et al. 1999; van Doorn et al. 2007; Yori et al. 2006). The three groups are as follows: (i) copro-diagnostic procedures with low sensitivity (12.9-68.9%); (ii) copro-diagnostic procedures with moderate sensitivity (47.1-96.8%); (iii) serological diagnostic procedures with high sensitivity (68.0-98.2%). Beta prior distributions were specified for the different diagnostic-test group sensitivities.

5.3.2.1 Estimating country-wide prevalence in the general population

The retrieved data was analyzed separately in the three different subsets: community-based studies, hospital-based studies, and studies on refugees and immigrants, as prevalence rates from these subsets cannot be directly compared.

Model-based prevalence estimates for each study type and country were plotted on a world map, using ArcGIS (version 9.3). The prevalence estimates for refugee and immigrant studies were displayed in the country where the study was undertaken and not in the country from where the refugees and immigrants originated.

5.3.3 Association with specific risk factors

To analyze the association between *S. stercoralis* and specific risk factors, namely HIV/AIDS, Human T-lymphotropic virus 1 (HTLV-1) infected individuals, diarrhea, and alcoholism, the studies were grouped into case-control studies and cross-sectional studies. We used case-control studies conducted on each risk group with complete information about individuals screened (tested) and infected with *S. stercoralis*, as well as the diagnostic method used, to model specific Odds Ratios (OR) and pool them into an overall estimate using a logistic model taking into account the prior information available on diagnostic test sensitivity.

5.3.4 Diagnostic test sensitivity

The Bayesian models employed in this study estimate the disease prevalence (or ORs) together with the diagnostic sensitivity. We run the models under different prior specifications, to assess the robustness of the estimates.

5.4 Results

5.4.1 Study Identification

We identified and reviewed 354 studies (Figure 5.2). Of those, 194 (54.8%) used a cross-sectional design and were conducted in communities: 121 (62.4%) used diagnostic methods with low sensitivity, 56 (28.9%) with moderate sensitivity, and 17 (8.8%) with high sensitivity. Out of 121 hospital-based studies, 75 (61.5%) used low, 36 (29.8%) used moderate and 10 (8.3%) used high sensitivity methods. Of the 39 studies on refugees and immigrants, 28 (71.8%) used low, three (7.7%) used moderate, and eight (20.5%) used high sensitivity diagnostic methods.
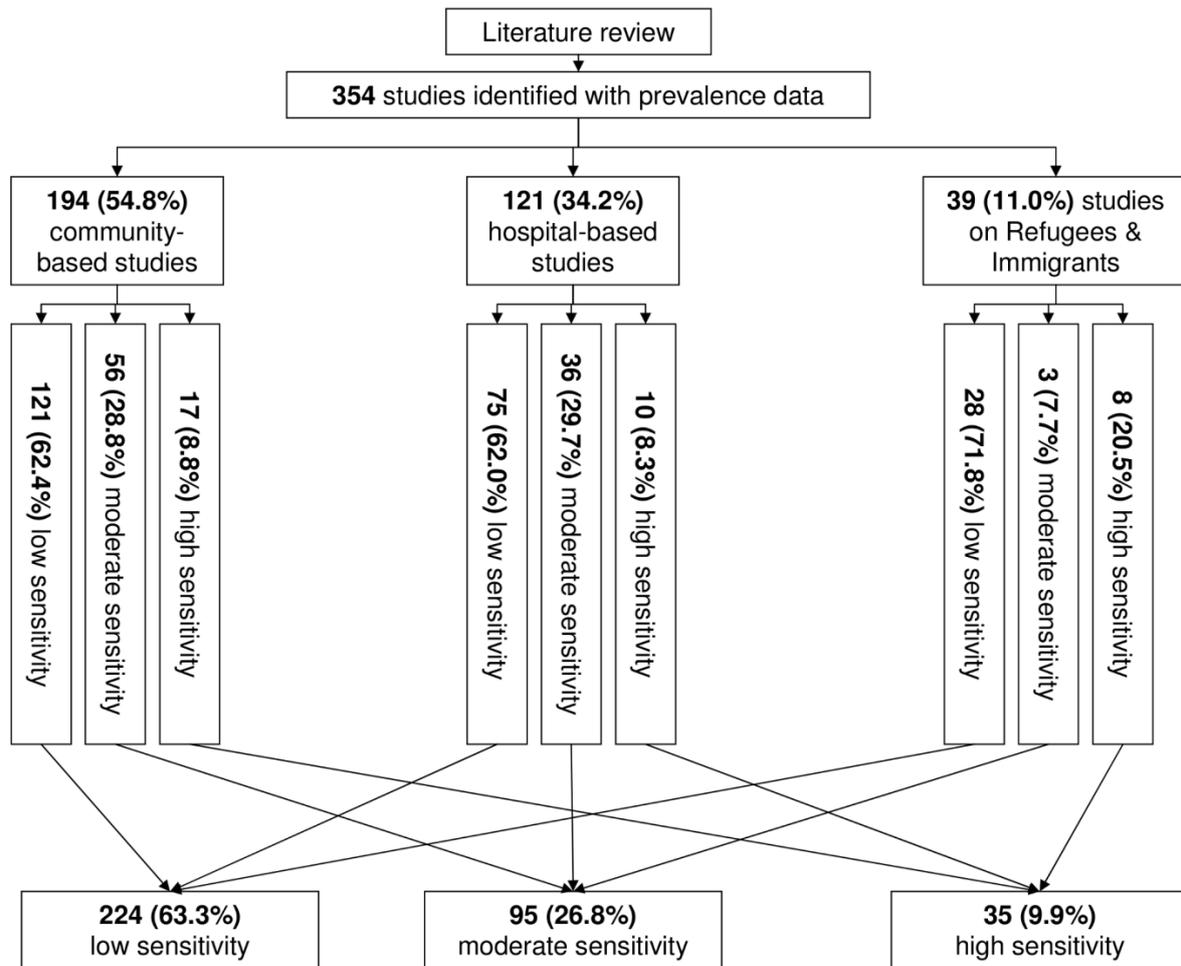
Figure 5.2 – Flow-chart of the literature review

5.4.2 Prevalence

5.4.2.1 Available information

Figure 5.3 indicates the number of reports per country that provided information on infection rates. Tables 5.1 – 5.3 report the calculated prevalence rates per country. Information is notably scarce for those African countries where environmental and socioeconomic conditions are most favorable for transmission. *S. stercoralis* infection data is only available for 20 (43.5%) of the 46 African countries. The distribution of infection rate information is heterogeneous. Almost a quarter of the studies (18, 23.4%) were undertaken in densely populated Nigeria alone. Some studies reported on tropical West and East Africa. However, infection rate data is scarce for Sahelian, Central and Southern Africa. Most of the available studies used low sensitivity diagnostic methods. Adequate diagnostic techniques, such as

the Baermann method and Koga Agar plate culture, were employed in only 19.0% of the studies in African countries.

The Americas are well covered, with studies in 21 (60.0%) of the 35 countries in this region. Data is mostly missing for smaller countries, such as the Caribbean island nations (Antigua, Barbuda, Bahamas, Barbados, etc.). A large amount of information is available for Brazil, where 43 (37.4%) studies were undertaken. Most investigations were conducted in communities (26, 60.5%) rather than in hospitals (16, 37.2%). For the United States of America, 22 (19.1%) studies were identified. Almost two thirds of them (14, 63.6%) focused on refugees and immigrants.

For Europe, comparably fewer reports (24) were found. Most of them focused on refugees, immigrants and travelers. South-East Asia and the Western Pacific region are reasonably represented, with 40 investigations conducted in Thailand (36.4%), 15 in Australia (13.6%), 14 in Japan (12.7%), and 14 in India (12.7%). Yet, in many other Asian countries where high prevalence of *S. stercoralis* is likely to occur, information on infection rates is limited, and studies often lack the use of high sensitivity methods.

5.4.2.2 Global prevalence of *S. stercoralis*

The global prevalence picture is as diverse and heterogeneous as the type and number of studies undertaken. The existing information suggests that *S. stercoralis* infections affect between 10% and 40% of the population in many tropical and subtropical countries. In resource-poor countries with ecological and socioeconomic settings conducive to the spread of *S. stercoralis*, high infection rates of up to 60% can be expected. The majority of the studies reviewed were undertaken at community-level (Figure 4). Yelifari and colleagues (Yelifari et al. 2005) conducted one of the biggest studies in Africa, in Northern Ghana, sampling 20,250 persons across 216 villages and therefore covering different settings. The infection rate was 11.6%. They found a slightly higher statistically significant infection rate in men (12.7%) than women (10.6%).

Studies based on health services data often focus on the number of patients reporting symptoms or suffering from conditions other than helminthiasis. If stool samples are analyzed, high sensitivity methods are only applied if the patient is suspected of having an intestinal parasitic infection, i.e. might be infected with *S. stercoralis*. A study from Guadeloupe (Nicolas et al. 2006) analyzed 17,660 hospital records from the university hospital in Pointe-à-Pitre, reporting 708 cases of *S. stercoralis* (4.0%). Yet in Guatemala, where 14,914 pregnant women were tested using a single stool sample and where low-sensitivity diagnostic methods were applied, the reported prevalence was as low as 0.4%

(Villar et al. 1989). This is an example for the difficulties comparing studies using different diagnostic approaches (Figure 5).

Studies on refugees and immigrants were mostly conducted, with a few exceptions, in developed countries (Figure 6). Most found high infection rates in immigrants and refugees, reaching prevalence rates of up to 75%. Infection rates varied substantially depending on the refugees' country of origin. In Canada in 1990, Gyorkos and colleagues (Gyorkos et al. 1990) used high sensitivity diagnostic tools and found a prevalence rate of 11.8% in Vietnamese refugees versus 76.6% in Cambodian refugees. In many countries, immigrants are routinely screened for helminthiasis if they attend a hospital. A study in Saudi Arabia by al-Madani and colleagues (al-Madani and Mahfouz 1995) analyzed 5,518 female housekeepers originating from different Asian countries. The overall prevalence reported was 0.6%; 0.4% in Filipinos, 0.5% in Indonesians, 1.5% in Sri Lankans, 2.6% in Indians and 3.4% in Thais, respectively.

5.4.2.3 Hotspots: Brazil and Thailand

Brazil and Thailand are *S. stercoralis* endemic countries where reliable and consistent data on infection is available. For Brazil, we found 43 studies (12.1% of all studies world-wide) that qualified for inclusion. Using data from the community-based studies, our model showed a prevalence of 13.0% (95% Bayesian Confidence Interval (BCI): 12.0-14.2%). The Baermann method was used in nine (34.6%) of these studies, and the Koga Agar plate culture in just four (15.4%). Analyzing data from the 16 hospital-based studies yielded a prevalence of 17.0% (95% BCI: 15.8-18.2%). The Baermann method was used in 15 (93.8%) studies, most often in combination with other methods, yet the Koga Agar plate culture was not used in any of the hospital-based studies in Brazil. Most hospital-based studies were undertaken in the big cities of Rio de Janeiro and São Paolo. Rossi and colleagues (Rossi et al. 1993) reported analyzing 37,621 laboratory specimens over a period of two years in the university hospital in the Campinas City region. The patients examined originated from all over Campinas City. The infection rate was estimated to be 10.8%.

In Thailand, a quarter to a third of the study participants tested positive for *S stercoralis.* In all studies conducted directly in the community, the overall prevalence was 23.7% (95% BCI: 21.8-26.1%). In contrast to Brazil, the main diagnostic approach used for the Thai studies was the Koga Agar plate culture, which was used in 10 (31.3%) of the studies. In hospitals (8, 20.0%), the infection prevalence was considerably higher and reached 34.7% (95% BCI: 31.6-38.3%). Five (62.5%) of these studies were undertaken in the capital Bangkok, four of which (50.0%) focused on HIV/AIDS-infected patients.

5.4.2.4 Other regional highlights and concerns

For Japan, all 14 studies were undertaken on the Okinawa islands. *S. stercoralis* is only endemic in Okinawa prefecture and the cases reported are mostly among older persons with sustained infection due to auto-infection. This was demonstrated in a study of Arakaki and colleagues (Arakaki et al. 1992a) which showed an overall infection rate of 16.4%; yet for individuals aged 10-39 years, the prevalence was only 5.5% whereas in individuals older than 40 years of age, the prevalence was 30.2%. Our country estimate of infection rates based on community data was 18.7% (95% BCI: 17.4-20.4%) and 13.6% (95% BCI: 12.7-14.5%) based on hospital investigations. All the studies from Japan employed a highly sensitive Koga Agar plate culture diagnostic method and often analyzed several stool samples per person. Arakaki and colleagues (Arakaki et al. 1992b) undertook a study of six different endemic regions in Okinawa, and reported a significant difference between infection rates in males (14.0%) and females (6.8%).

European studies principally focused on refugees, immigrants and travelers to endemic countries. A good example of this is found in a recent report on two Italian tourists returning from Southeast-Asia, presenting acute strongyloidiasis (Angheben et al. 2011). As an exception, in a study from Spain (Roman-Sanchez et al. 2003), infections were reported in farm workers in Gandia (south of Valencia, eastern Spain). The Koga Agar plate culture was used on three stool samples taken on consecutive days to diagnose a threadworm infection. Of the 250 farm workers, 12.4% were *S. stercoralis* positive. When adjusted for the sensitivity of the diagnostic method, our model found a prevalence of 14.8% (95% BCI: 10.3-20.3%). Another study from Gill and colleagues (Gill et al. 2004) of World War II veterans undertaken in 2004 in the United Kingdom showed that *S. stercoralis* infection might be sustained over a long time. Most participants had not left the UK since returning from their deployment in Southeast Asia and were evaluated some 60 years later. The study reported 248 cases from 2,072 veterans screened for *S. stercoralis* (12.0%); the adjusted prevalence was 12.7% (95% BCI: 11.1-14.5%).

Little information is available from countries with the largest populations, namely China and India. Studies on Mainland China are scarce or could not be included due to the language limitations of this review. Our calculation from a study of communities in Yunnan province resulted in a prevalence of 14.0% (95% BCI: 9.0-20.4%). The three other studies identified were conducted on immigrants, mainly from South-East Asian countries, working in Taiwan and presented an infection prevalence of 17.1% (95% BCI: 15.2-19.2%). For India, 14 studies were identified, nine of which were conducted on hospitalized persons, and reporting an infection rate of 11.2% (95% BCI: 8.6-14.4%). Five of these reports focus on HIV/Aids
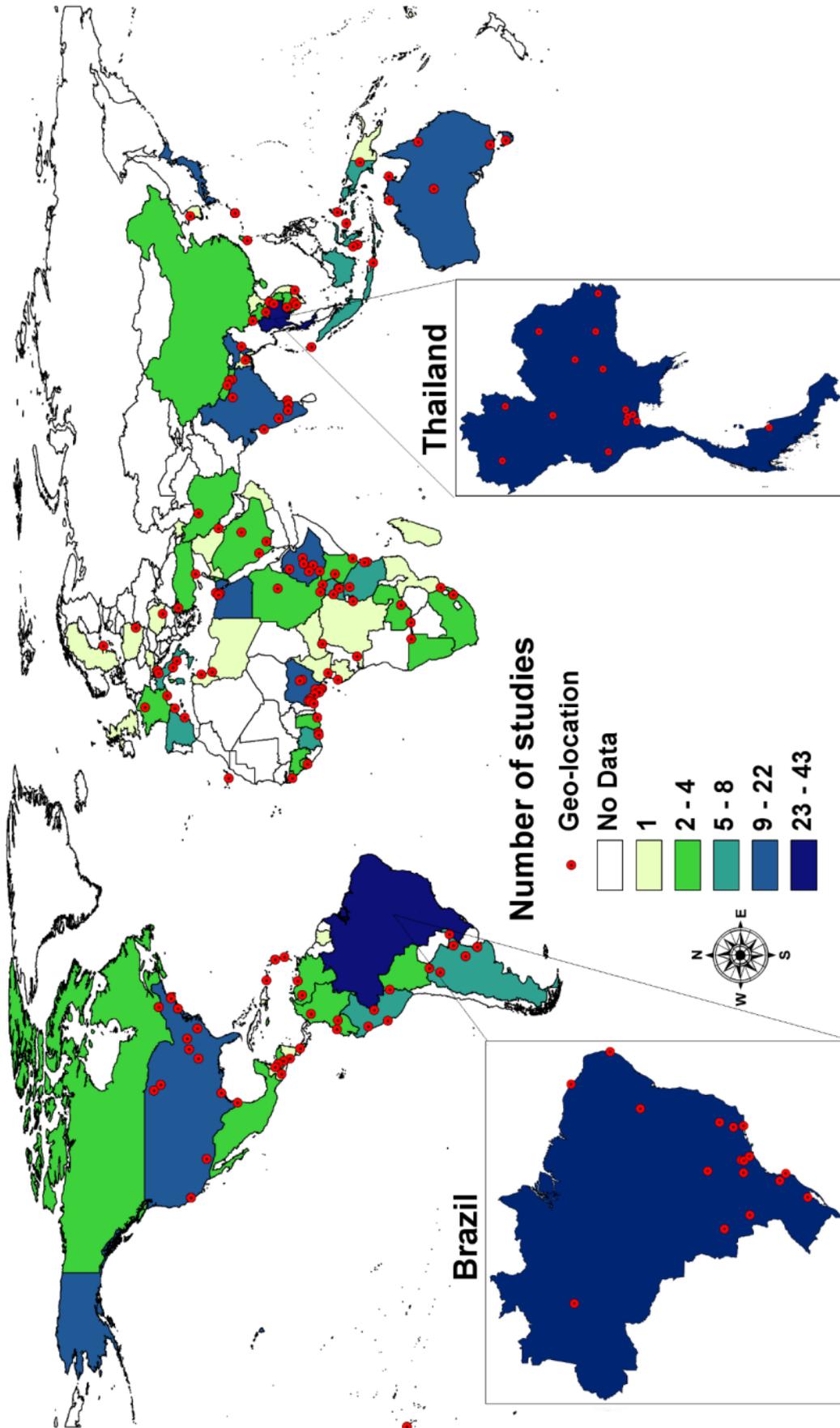
Figure 5.3 – Number of studies undertaken per country since 1989
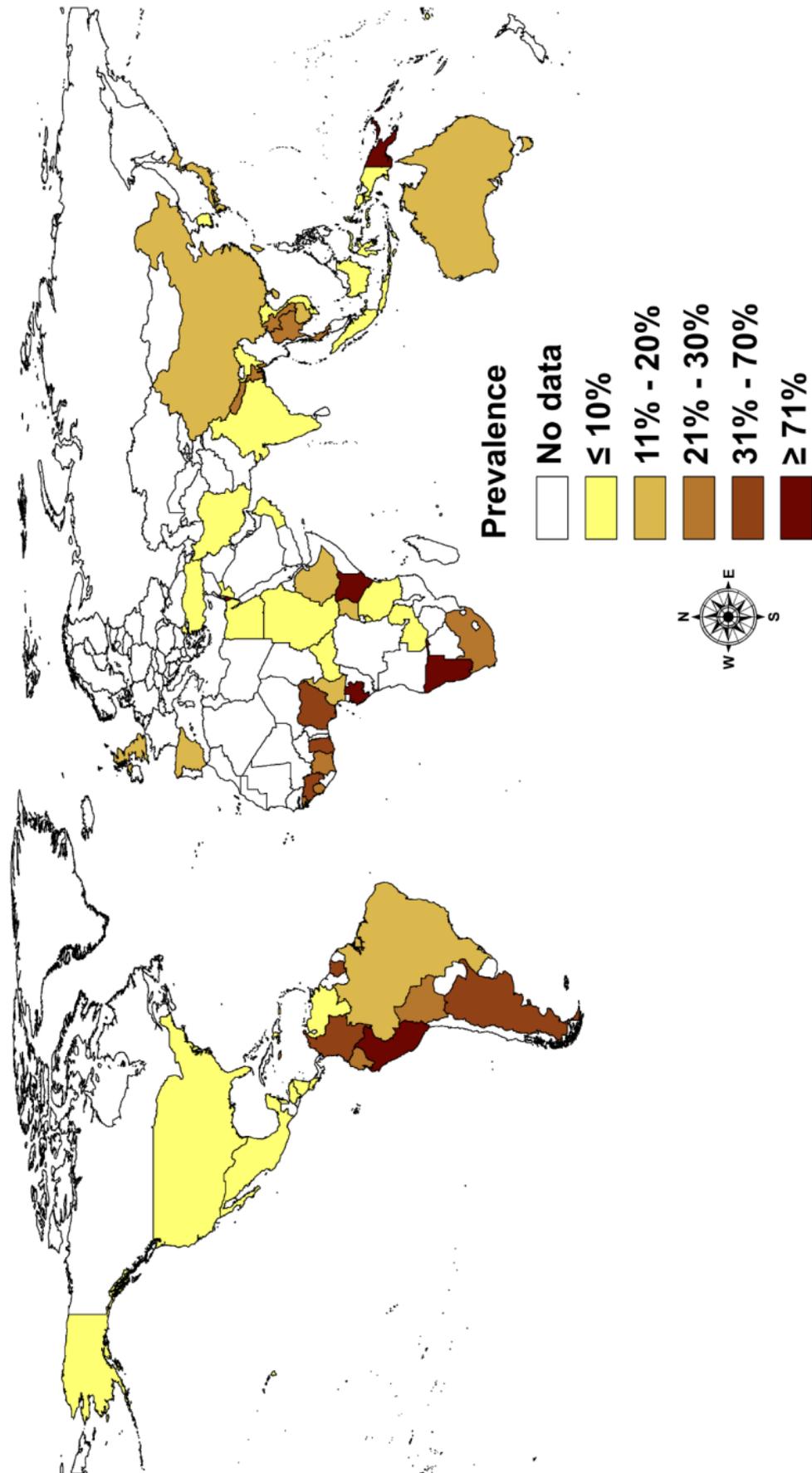
Figure 5.4 – Prevalence of *S. stercoralis* infection, based on community-based studies
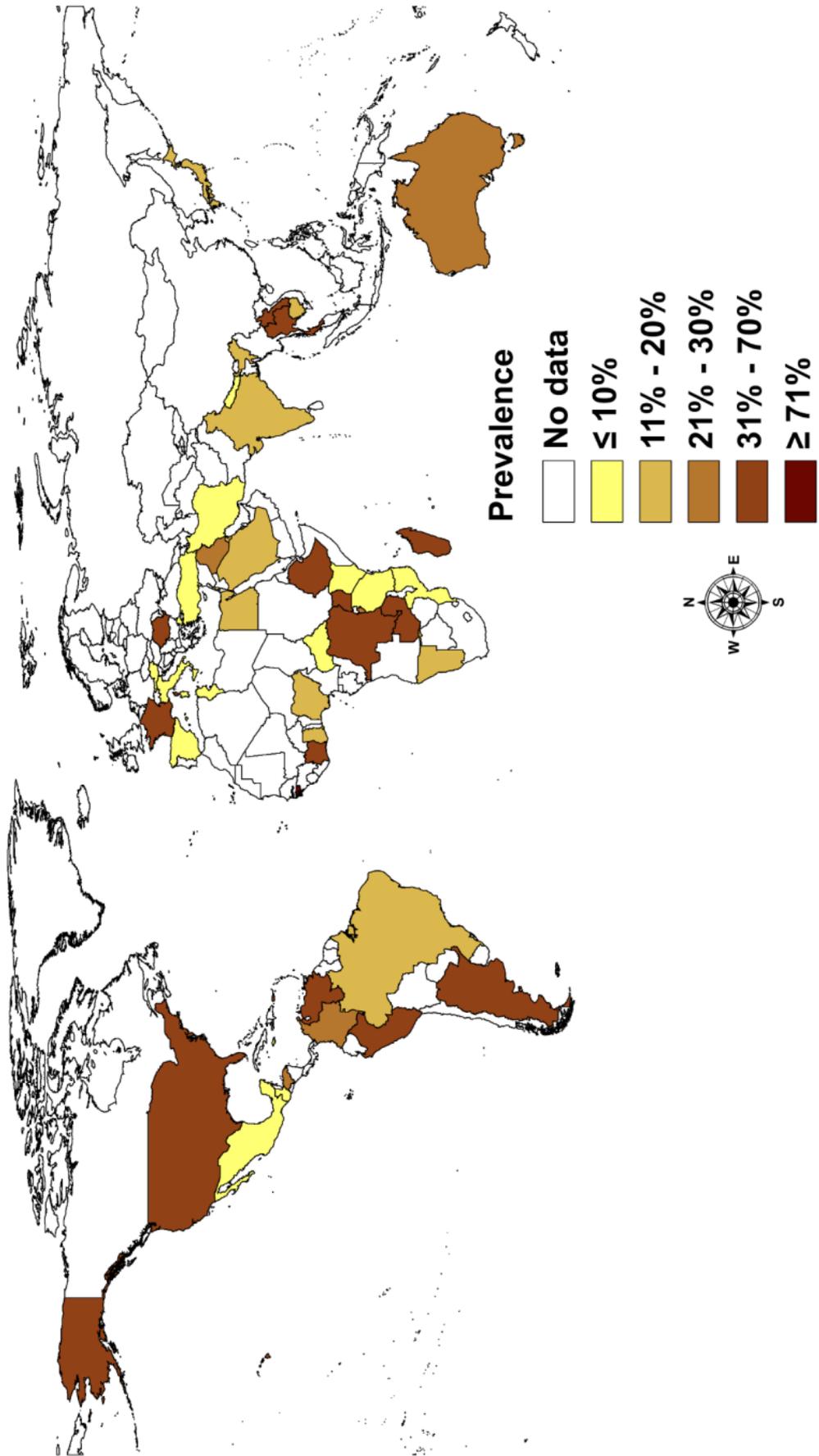
Figure 5.5 – Prevalence of *S. stercoralis* infection, based on health services studies
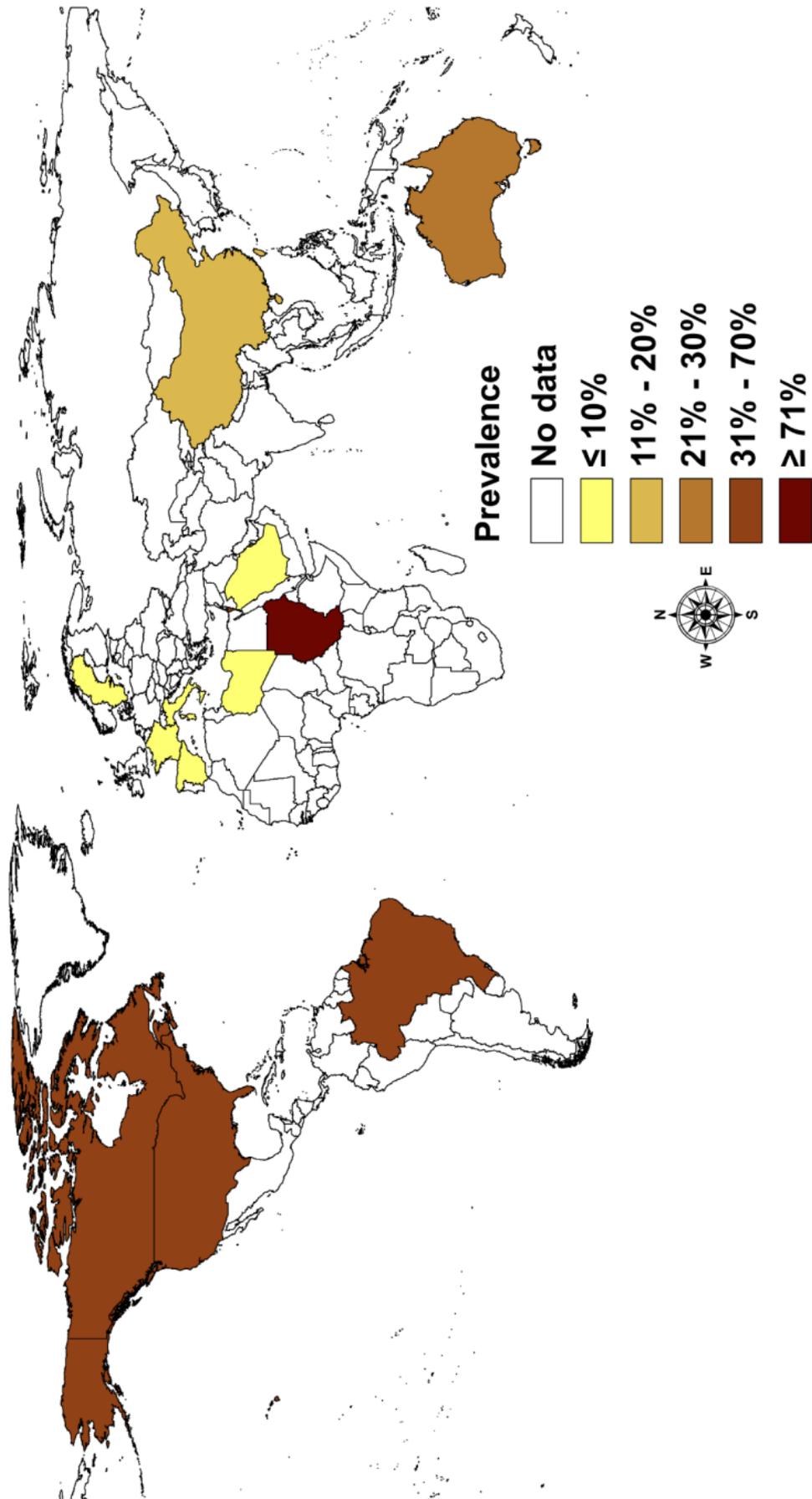
Figure 5.6 – Prevalence of *S. stercoralis* in refugees and immigrants

Table 5.1 – Country-wide prevalence for *S. stercoralis* (A-F), divided by type of study

Country	Total Number of surveys for prevalence calculation	Community-based surveys			Hospital-based surveys			Refugees & Immigrants		
		Total Number	Prevalence	95% CI	Total Number	Prevalence	95% CI	Total Number	Prevalence	95% CI
Argentina	8	4	52.8%	42.42% - 64.6%	4	63.0%	53.6% - 72.9%			
Australia	15	6	15.0%	13.52% - 16.8%	3	28.9%	26.2% - 31.6%	6	25.3%	22.3% - 28.5%
Austria	1				1	5.2%	1.0% - 15.7%			
Bangladesh	1	1	29.8%	21.7% - 39.8%						
Belize	1	1	7.7%	3.3% - 14.8%						
Bolivia	2	2	21.1%	11.2% - 36.1%						
Brazil	43	26	13.0%	12.0% - 14.2%	16	17.0%	15.8% - 18.2%	1	35.0%	9.8% - 85.4%
Burundi	2	1	1.9%	0.4% - 5.6%	1	21.6%	11.2% - 36.4%			
Cambodia	4	3	17.5%	15.7% - 19.6%	1	13.9%	12.1% - 16.0%			
Cameroon	1	1	10.0%	3.6% - 21.2%						
Canada	3							3	67.5%	61.3% - 73.5%
Central African Republic	2	1	0.1%	0.0% - 1.2%	1	1.9%	0.4% - 5.5%			
China	4	1	14.0%	9.0% - 20.4%				3	17.1%	15.2% - 19.2%
Colombia	2	1	56.2%	48.0% - 65.7%	1	20.2%	6.7% - 45.1%			
Costa Rica	1	1	6.9%	2.9% - 13.6%						
Côte d'Ivoire	5	4	24.3%	20.7% - 28.4%	1	67.7%	41.4% - 98.7%			
DR of the Congo	1				1	32.7%	20.6% - 48.6%			
Dominica	1	1	97.6%	78.4% - 100%						
Ecuador	2	2	27.1%	19.3% - 36.9%						
Egypt	12	5	2.5%	2.0% - 3.2%	7	11.1%	9.4% - 13.1%			
Ethiopia	12	7	15.9%	14.1% - 17.9%	5	31.0%	23.6% - 40.0%			
Fiji	1	1	9.3%	2.5% - 23.1%						
France	2				1	31.1%	22.7% - 40.7%	1	5.6%	3.7% - 8.9%

Table 5.2 – Country-wide prevalence for *S. stercoralis* (G-M), divided by type of study

Country	Total Number of surveys for prevalence calculation	Community-based surveys			Hospital-based surveys			Refugees & Immigrants		
		Total Number	Prevalence	95% CI	Total Number	Prevalence	95% CI	Total Number	Prevalence	95% CI
Gabon	1	1	91.8%	44.6% - 100.0%						
Ghana	2	1	69.5%	63.2% - 76.9%	1	13.6%	1.1% - 53.4%			
Grenada	1	1	3.3%	0.3% - 13.0%						
Guadeloupe	2	1	18.7%	14.5% - 23.5%	1	8.3%	7.7% - 8.9%			
Guatemala	1				1	2.0%	1.5% - 2.6%			
Guinea	2	2	43.8%	34.7% - 54.9%						
Guinea-Bissau	2	1	23.7%	18.3% - 30.0%	1	84.2%	42.1% - 100.0%			
Haiti	1	1	1.0%	0.5% - 1.8%						
Honduras	4	1	3.2%	1.5% - 6.2%	3	29.8%	24.1% - 36.0%			
India	14	5	6.6%	4.4% - 9.4%	9	11.2%	8.6% - 14.4%			
Indonesia	6	6	7.6%	6.2% - 9.3%						
Iran	3	1	0.3%	0.1% - 0.5%	2	0.6%	0.1% - 1.7%			
Iraq	1				1	24.2%	14.1% - 38.1%			
Israel	3	1	94.9%	86.4% - 100.0%	4	1.8%	1.4% - 2.3%	2	31.0%	27.0% - 35.1%
Italy	5	5	27.1%	22.8% - 32.1%	1	1.8%	0.9% - 3.2%	1	3.3%	0.6% - 9.6%
Jamaica	3	2	18.7%	17.4% - 20.4%						
Japan	14	9	0.03%	0.0% - 0.1%	5	13.6%	12.7% - 14.5%			
Jordan	1	1								
Kenya	4	2	80.2%	61.1% - 99.4%	2	7.8%	5.0% - 11.5%			
Kuwait	1				1	16.3%	14.1% - 18.7%			
Lao PDR	4	3	26.2%	22.5% - 30.4%	1	55.8%	37.0% - 81.4%			
Libya	1							1	1.1%	0.1% - 4.5%
Madagascar	1				1	52.2%	42.6% - 61.6%			
Martinique	2	1	3.8%	3.3% - 4.4%	1	9.6%	8.1% - 11.4%			
Mexico	2	1	1.6%	0.2% - 6.3%	1	5.7%	1.1% - 16.5%			
Mozambique	1				1	6.2%	2.5% - 12.1%			

Table 5.3 – Country-wide prevalence for *S. stercoralis* (N-Z), divided by type of study

Country	Total Number of surveys for prevalence calculation	Community-based surveys			Hospital-based surveys			Refugees & Immigrants		
		Total Number	Prevalence	95% CI	Total Number	Prevalence	95% CI	Total Number	Prevalence	95% CI
Namibia	3	2	99.3%	92.2% - 100.0%	1	14.3%	11.6% - 17.6%			
Nepal	3	1	22.8%	10.1% - 43.4%	2	5.8%	2.5% - 11.2%			
Nicaragua	1	1	2.0%	0.6% - 4.5%						
Nigeria	18	13	48.1%	43.3% - 53.8%	5	17.6%	15.2% - 20.3%			
Occ. Palestinian Territ.	1				1	4.2%	0.4% - 16.7%			
Oman	1	1	3.0%	0.6% - 8.7%						
Papua New Guinea	1	1	99.0%	90.0% - 100.0%						
Peru	6	4	75.3%	70.8% - 82.0%	2	69.3%	61.1% - 77.9%			
Puerto Rico	2	1	16.0%	3.0% - 47.5%	1	33.5%	13.7% - 66.6%			
Republic of Korea	2	2	0.1%	0.0% - 0.1%						
Romania	1				1	48.8%	31.1% - 72.1%			
Saint Lucia	1	1	58.5%	44.1% - 76.4%						
Saudi Arabia	3				1	12.5%	3.3% - 31.2%	2	7.1%	5.5% - 9.0%
Sierra Leone	3	3	27.4%	21.5% - 34.4%						
South Africa	2	2	27.5%	21.3% - 34.7%						
Spain	5	1	14.8%	10.3% - 20.3%	1	1.9%	1.6% - 2.2%	3	4.2%	2.8% - 6.1%
Sudan	3	2	3.7%	1.9% - 6.4%				1	98.9%	89.2% - 100.0%
Suriname	1	1	63.2%	50.3% - 78.2%						
Sweden	1							1	1.0%	0.4% - 2.1%
Thailand	40	32	23.7%	21.8% - 26.1%	8	34.7%	31.6% - 38.3%			
Tunisia	1				1	0.5%	0.3% - 0.9%			
Turkey	3	1	0.6%	0.4% - 0.8%	2	4.1%	2.1% - 7.2%			
Uganda	6	4	19.3%	17.1% - 21.9%	2	30.3%	25.1% - 36.5%			
UK	1	1	12.7%	11.1% - 14.5%						
UR of Tanzania	8	4	7.9%	6.6% - 9.5%	4	9.3%	6.1% - 13.7%			
US of America	22	3	2.7%	2.4% - 3.0%	5	49.2%	0.1% - 99.9%	14	40.4%	37.8% - 43.0%
Venezuela	3	1	2.3%	0.2% - 9.1%	2	48.4%	0.2% - 99.8%			
Viet Nam	1	1	0.02%	0.0% - 0.3%						
Zambia	3	1	6.6%	1.3% - 19.4%	2	50.6%	0.2% - 99.9%			

patients. For the five community-level studies, an infection rate of 6.6% was reported (95% BCI: 4.4-9.4%). For other countries with large populations, such as Indonesia, Pakistan and Bangladesh, which combined account for over half a billion inhabitants, only seven studies were available (Indonesia: 6, Bangladesh: 1, Pakistan: 0). All seven studies were conducted at community-level, and infection rates of 7.6% (95% BCI: 6.2-9.3%) in Indonesia and 29·8% (95% BCI: 21.7-39.8%) in Bangladesh, respectively, suggest a considerable burden of infection in these populous countries.

5.4.3 High risk groups for Strongyloides stercoralis infection

5.4.3.1 HIV/AIDS patients

Many countries with high HIV-prevalence rates are also highly *S. stercoralis* endemic, and co-infection may occur. *S. stercoralis* no longer constitutes an AIDS-defining, opportunistic infection (WHO 2007) as it did during the onset of the HIV-pandemic. For 29 cross-sectional studies focusing on HIV-positive individuals, we calculated *S. stercoralis* prevalence rates per country. The rates varied substantially from 1.0% (95% BCI: 0.0-2.0%) in Iran to as high as 43.0% (95% BCI: 20.0-83.0%) in Ethiopia. The overall prevalence for HIV-positive individuals was 10.0% (95% BCI: 5.0-20.0%). We identified 16 case-control studies comparing HIV-positive individuals with sero-negative controls.

HIV+	Sensitivity	Cases n/N	Controls n/N	OR (95% BCI)	OR (95% BCI)
Moura H et al. (1989)	Moderate	15/99	17/260		2.70 (1.32 − 5.58)
Floch PJ et al. (1989)	Moderate	10/100	2/137		3.76 (1.43 − 11.3)
Conlon CP et al. (1990)	Moderate	3/44	0/36		2.36 (0.63 − 9.61)
Hunter G et al. (1992)	Low	2/90	1/105		1.60 (0.38 − 6.05)
Dias RM et al. (1992)	Moderate	54/554	15/142		1.18 (0.65 − 2.15)
Mendez OC et al. (1994)	Low	2/82	8/300		1.25 (0.33 − 3.75)
Gomez Morales MA et al. (1995)	Low	11/112	3/239		4.98 (1.94 − 14.7)
Lindo JF et al. (1998)	Low	4/52	0/48		3.14 (0.89 − 13.3)
Lebbad M et al. (2001)	Low	5/37	3/15		2.48 (0.70 − 9.57)
Feitosa G et al. (2001)	Moderate	20/365	38/5243		6.71 (3.75 − 11.5)
Marchi BJ et al. (2003)	Moderate	21/211	12/213		1.98 (1.00 − 3.98)
Adjei A et al. (2003)	Low	1/21	0/27		2.13 (0.42 − 9.86)
Hailemariam G et al. (2004)	Low	4/78	1/26		1.81 (0.50 − 6.76)
Silva CV et al. (2005)	Moderate	12/100	6/85		2.10 (0.87 − 5.18)
Pinlaor S et al. (2005)	Moderate	14/78	6/78		3.01 (1.26 − 7.70)
Getaneh A et al. (2010)	Low	4/192	23/192		0.34 (0.11 − 0.89)
Total					2.17 (1.18 − 4.01)

Figure 5.7 – Risk of *S. stercoralis* infection in HIV/AIDS patients

Four reported a lower or similar prevalence in the two groups (Dias et al. 1992; Fontanet et al. 2000; Lebbad et al. 2001; Mendez et al. 1994). All other studies showed an increased *S. stercoralis* infection risk for HIV-positive individuals; three showed a statistically significant risk (Feitosa et al. 2001; Gomez Morales et al. 1995; Pinlaor et al. 2005). Our meta-analysis resulted in a pooled OR of 2.17 (95% BCI: 1.18–4.01) for HIV-positive individuals (Adjei et al. 2003; Conlon et al. 1990; Dias et al. 1992; Feitosa et al. 2001; Floch et al. 1989; Getaneh et al. 2010; Gomez Morales et al. 1995; Hailemariam et al. 2004; Hunter et al. 1992; Lebbad et al. 2001; Lindo et al. 1998; Marchi Blatt and Cantos 2003; Mendez et al. 1994; Moura et al. 1989; Pinlaor et al. 2005; Silva et al. 2005) (Figure 5.7) compared to the HIV-negative controls.

5.4.3.2 HTLV-1 patients

Persons infected with human T-lymphotropic virus 1 (HTLV-1) tend to be significantly co-infected with *S. stercoralis* in comparison with HTLV-1-seronegative controls (Hayashi et al. 1997; Marsh 1996; Phelps 1993; Robinson et al. 1994). Our meta-analysis resulted in a pooled OR of 2.48 (95% BCI: 0.70-9.03) for the infection with HTLV-1 (Chieffi et al. 2000; Courouble et al. 2004; Einsiedel and Woodman 2010; Nera et al. 1989) (Figure 5.8), showing no statistically significant difference.

HTLV−1	Sensitivity	Cases n/N	Controls n/N	OR (95% BCI)	OR (95% BCI)
Nera FA et al. (1989)	High	27/94	27/106		1.44 (0.73 − 2.70)
Chieffi PP et al. (2000)	Moderate	11/91	1/85		2.92 (1.08 − 11.6)
Courouble G et al. (2004)	High	27/61	33/255		4.95 (2.47 − 11.0)
Einsiedel LJ et al. (2010)	High	37/78	239/675		1.92 (1.12 − 3.52)
Total					2.48 (0.70 − 9.03)
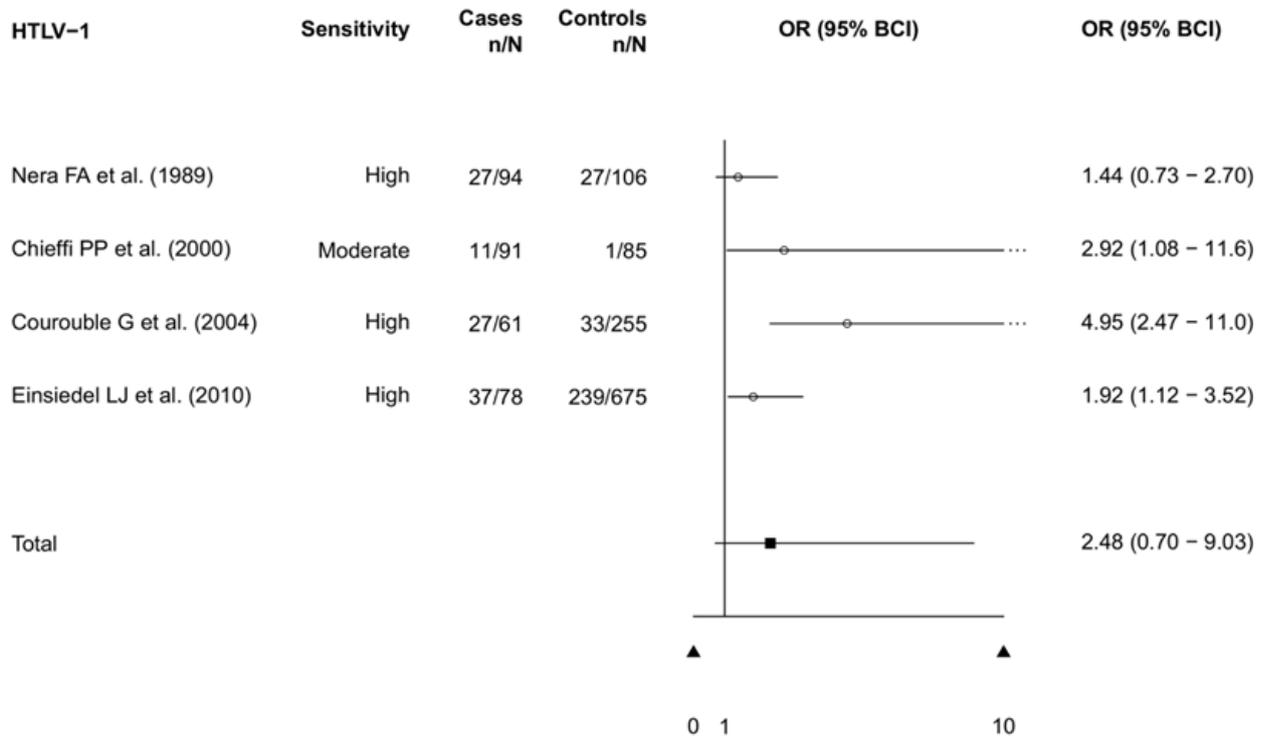
Figure 5.8 – Risk of *S. stercoralis* infection in patients with HTLV-1 infection

In HTLV-1 infected patients, eradication of the parasite by conventional drug therapy is hindered (Satoh et al. 2002). *S. stercoralis* hyperinfection syndrome, including its fatal outcome, is particularly common in these patients (Gotuzzo et al. 1999). *S. stercoralis* co-infection appears to shorten the latency period until the onset of adult T-cell leukaemia in HTLV-1 positive subjects (Plumelle et al. 1997).

5.4.3.3 Alcoholics

Four studies (three case-control studies, and one cross-sectional study) focused on patients with an alcohol addiction. The case-control studies, all from Brazil, showed higher infection rates in alcoholics than in the control groups (de Oliveira et al. 2002; Gaburri et al. 1997; Zago-Gomes et al. 2002). The meta-analysis resulted in a pooled OR of 6.69 (95% BCI: 1.47-33.8, Figure 9). The study by Zago-Gomes and colleagues (Zago-Gomes et al. 2002) showed that only *S. stercoralis* infection rates differed between alcoholics and control groups. Contrastingly, other nematodes showed the same prevalence in alcoholics and control groups. Zago-Gomes and colleagues argue that alcoholics' regular ethanol intake might lead to an immune modulation and/or alteration in corticosteroid metabolism, favoring *S. stercoralis* infection.

Alcoholism	Sensitivity	Cases n/N	Controls n/N	OR (95% BCI)	OR (95% BCI)
de Oliveira LC et al. (2002)	Moderate	18/55	5/90		7.97 (3.72 − 27.0)
Zago−Gomes MP et al. (2002)	Moderate	43/198	28/440		4.97 (2.85 − 8.84)
Marques CC et al. (2010)	Low	54/263	26/590		8.43 (4.41 − 88.1)
Total					6.69 (1.47 − 33.8)
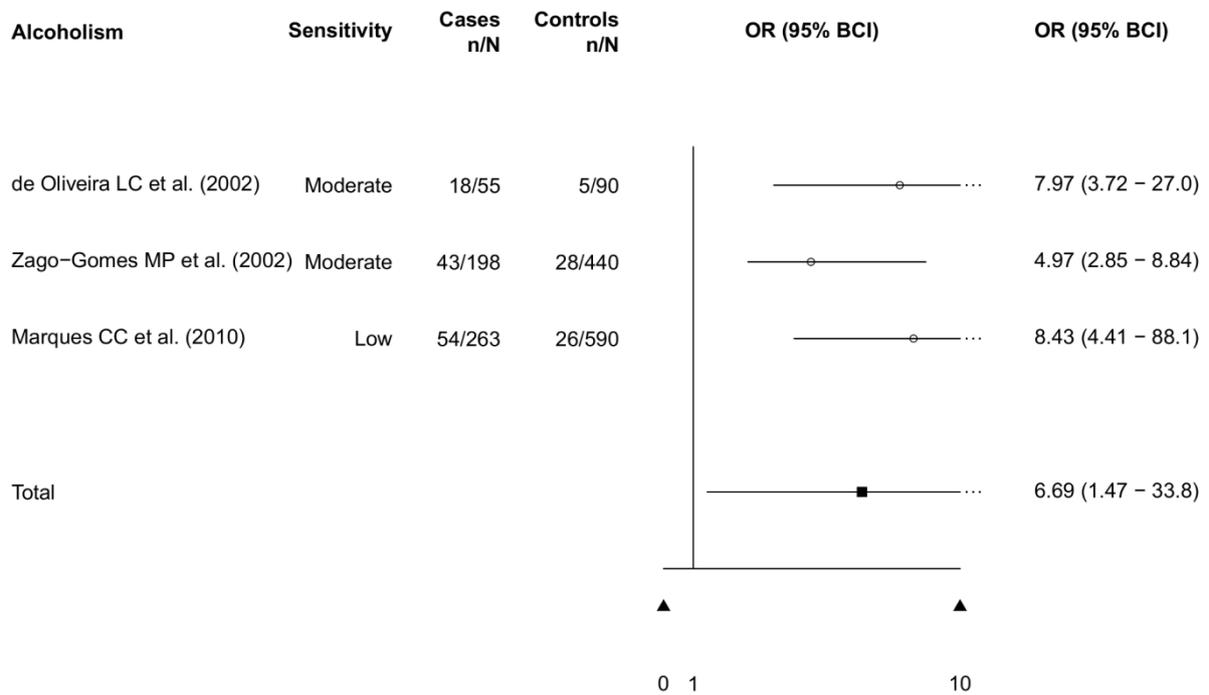
Figure 5.9 – Risk of *S. stercoralis* infection in alcoholics

5.4.3.4 Patients with diarrhea

Studies undertaken in patients with diarrhea showed a wide range of infection prevalences. The lowest infection rate was 1.0% (95% CI: 0.0-3.0%) found in a tertiary care hospital in Andhra Pradesh in India (Nagamani and Rajkumari 2001), while the highest reported was 76.0% (95% CI: 39.0-99.0%) in a study on Cambodian children in a refugee camp at the Thai-Cambodian border (Boyajian 1992). Comparing case-control studies lead to a pooled OR of 1.82 (95% BCI 0.19-12.2), showing no statistically significant difference (Henry et al. 1995; Hoge et al. 1995; Kukuruzovic et al. 2002; Molbak et al. 1994). Case-control studies on patients with and without diarrhea are relatively scarce, especially studies reporting on *S. stercoralis,* of which we could only identify four. Because diarrhea is one of the symptoms associated with *S. stercoralis* infection, as well as with other STH-infections, it remains unclear whether diarrhea can be considered as a risk factor, or if infection with STHs leads to a higher prevalence of diarrhea (Figure 10).

Diarrhea	Sensitivity	Cases n/N	Controls n/N	OR (95% BCI)	OR (95% BCI)
Molbak K et al. (1994)	Low	54/1219	15/511		1.80 (0.99 − 3.44)
Henry MC et al. (1995)	Low	17/173	3/155		6.64 (2.06 − 60.2)
Hoge CW et al. (1995)	Low	0/124	1/103		0.23 (0.00 − 2.39)
Kukuruzovic R et al. (2002)	Low	21/291	2/84		3.81 (1.40 − 13.8)
Total					1.82 (0.19 − 12.2)

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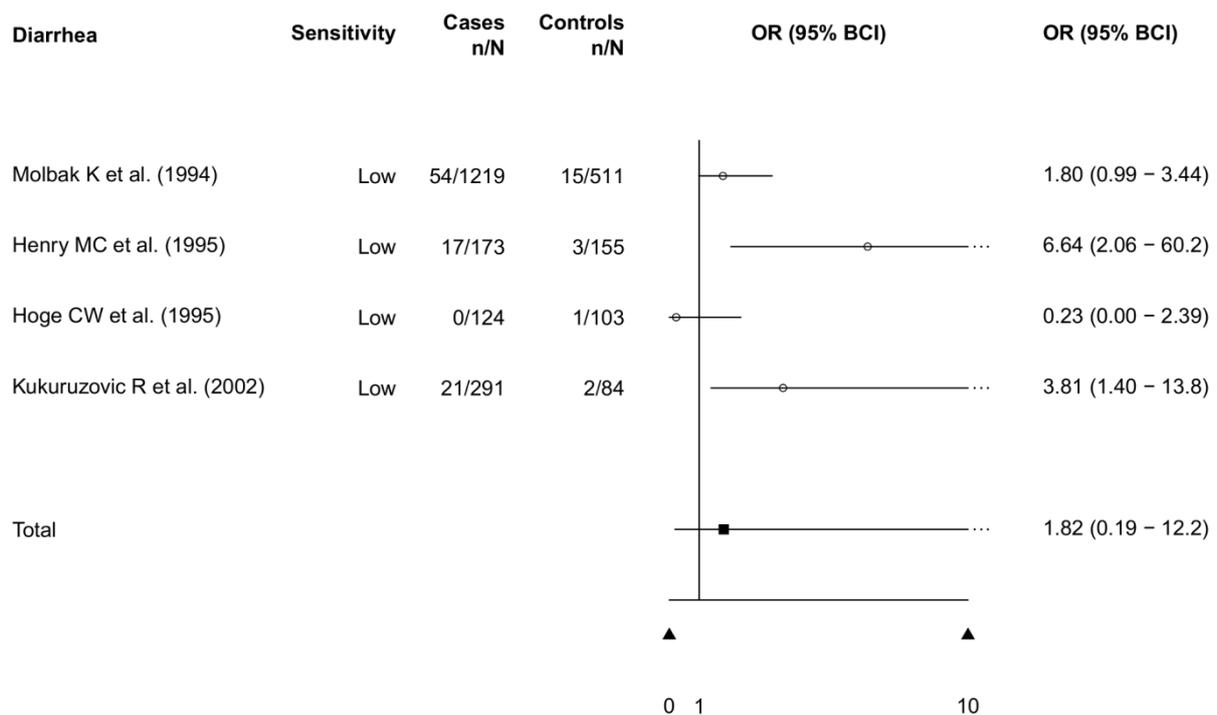
Figure 5.10 – Risk of *S. stercoralis* infection in patients with diarrhea

5.4.3.5 Patients with malignancies and/or immuno-compromising conditions

Case-control studies often focus on the infestation rates among patients with haematologic neoplastic diseases and/or immuno-suppressing conditions, arising, for instance, as a consequence of treatment. Two studies from Egypt show that *S. stercoralis* is found more

often in patients with malignant diseases undergoing immuno-suppressive treatment (Abaza et al. 1995; Khalil et al. 1991). In Japan, Hirata and colleagues (Hirata et al. 2007) found the parasite more often in patients diagnosed with biliary tract or pancreatic cancer. The infection rate was 7.5% among the 1,458 controls, 18.4% in the biliary tract cancer group, and 15.4% in the pancreatic cancer group. The liver cancer group reported the same infection rate (7.5%) of strongyloidiasis as the control group. One case-control study from Brazil found *S. stercoralis* to be more prevalent in immuno-compromised children in comparison with an immuno-competent control population by using serological techniques only. Four different serological approaches were used, each reporting higher infection rates in immuno-compromised children (e.g. ELISA-IgG: 12.1% versus 1.5%) than in the control group. No differences could be demonstrated (2.4% versus 4.4%) when based on parasitological examinations of stool samples, using the Baermann method, for three consecutive days (de Paula et al. 2000).

The malignancies and immuno-comprising conditions reported in the literature are manifold, leading to a very heterogeneous set of data. This makes meta-analysis virtually impossible.

5.4.3.6 Children

Of the 354 studies, 84 (23.7%) were conducted specifically on children, adolescents and young adults (aged 0-20 years). One third of them 29 (34.5%) were conducted in Africa, followed by 22 (26.2%) in the Americas and 19 (22.6%) in South-East Asia. The Western Pacific region (9), Middle East (4) and Europe (1) make up the remaining 14 (16.7%) studies. Almost all of these studies are cross-sectional and focus on children only.

Seven studies compared children with adults, but their comparison is challenged by very heterogeneous age grouping and matching. Two studies were conducted in Indonesia; Mangali and colleagues (Mangali et al. 1994) reported a prevalence of 4.4% in the group aged 2-14 years, and 6.7% in all participants aged 15 or older. The study by Toma and colleagues (Toma et al. 1999) reported similar trends with a prevalence of 0% in the group aged 4-14 years and 1.2% in all participants aged 15 years or older. The study by Dancesco and colleagues (Dancesco et al. 2005) in Côte d'Ivoire presented a prevalence of 12.2% in children aged 4-15 years, and 17.7% in adults, also underlining the trend of children having lower prevalence rates than adults. In contrast, the study by Gaburri and colleagues (Gaburri et al. 1997) showed a prevalence of 1.9% in adults, and 13.2% in children. The Gaburri study, however, focused on hepatic cirrhosis patients, and the prevalence rates are derived from only partially matched control groups. In Nepal, the study by Navitsky and colleagues (Navitsky et al. 1998) found a prevalence of 2.0% in 292 pregnant women (aged 15-40 years) and 0% in 129 infants (aged 10-20 weeks). The study by Wongjindanon and colleagues (Wongjindanon et al. 2005) found a prevalence of 9.7% in adult volunteers in

Surin (rural), Thailand, while the prevalence in schoolchildren from Samut Sakhon (suburban) was 2.0%. Due to the heterogeneity of the reported data, meta-analysis was not performed.

5.4.4 Diagnostic test sensitivity estimation

Estimates were robust to the prior specification, however they varied among the different study types. Hospital-based surveys led to higher sensitivity estimates than the community-based ones. Sensitivity estimates in the low sensitivity group range from 0.15 to 0.18 in the community-based surveys and from 0.17 to 0.21 in the hospital-based surveys. Sensitivity in the moderate sensitivity group is estimated between 0.77 and 0.90 in the community-based surveys. Higher uncertainty is observed in the estimation of the same diagnostic tools in hospital-based surveys, probably due to a smaller sample sizes. Sensitivity estimates in serological tests vary between 0.88 and 0.98 in community-based studies whereas they are more precise in the hospital-based surveys (0.94-0.98). The meta-analysis included limited number of surveys on immigrants and therefore the corresponding sensitivity estimates can not be compared to those from community- or hospital-based surveys.

5.5 Discussion

5.5.1 Prevalence rates of S. stercoralis

World-wide prevalence rates of *S. stercoralis* have been estimated on several occasions. Values vary from three million to one-hundred million infected individuals (Genta 1989; Jorgensen et al. 1996; Mahmoud 1996; Polenakovik 2004; Schwartz 2007). In 1989, after having examined the epidemiological evidence, Genta (Genta 1989) called these estimates "little more than inspired guesses" and cast doubts on the "practical value" of those numbers. In fact, knowledge on country and regional *S. stercoralis* infection rates and risks in specific population groups is of increasing clinical and epidemiological importance. Infected individuals are at risk of developing complicated strongyloidiasis as soon as cell-mediated immunity is compromised. The widespread and increasing use of corticosteroids for immuno-suppressive treatment, especially in *S. stercoralis* endemic areas, exacerbates the risk for severe complications associated with this infection.

Our findings provide an overview of the global prevalence of *S. stercoralis,* drawn from published infection reports since 1989. For the first time, we report prevalence rates on a country-by-country basis, based on published infection rates and taking into account the sensitivity of the diagnostic methods used. In Africa, the range of infection rates in the communities varies from 0.1% in the Central African Republic to up to 91.8% in Gabon. In

South- and Central-America, Haiti reports a prevalence of 1.0%, while in Peru the infection rate is as high as 75.3%. Interestingly, in South-East Asia, another highly endemic part of the world, several countries report infection rates within a comparably small range. In Cambodia, the infection rate is 17.5%, Thailand 23.7% and Lao PDR 26.2%. Only Vietnam, with a prevalence of 0.02% - based on only one study - falls out of this picture.

In general, information on infection rates/prevalence of the parasite is scarce, and the studies we analyzed suggest that infection with *S. stercoralis* is highly underreported, especially in Sub-Saharan Africa and Southeast Asia. The main reason is that almost no studies focusing on *S. stercoralis* were conducted. Therefore, studies reporting *S. stercoralis* prevalence most often used low-sensitivity diagnostic methods for *S. stercoralis* and only samples from one day were analyzed. Furthermore, information about at-risk groups and affected populations is missing, as few studies focus on strongyloidiasis and possible at-risk groups.

S. stercoralis has a very low prevalence in societies where fecal contamination of soil is rare. Hence, it is a very rare infection in developed countries and is less prevalent in urban than in rural areas of resource poor countries, with the exception of slum areas in the bigger cities. In Europe and in the United States the infection occurs in pockets and predominantly affects individuals pursuing farming activities or miners. In Germany, *S. stercoralis* is recognized as a parasitic professional disease in miners (Arbeitsmedizin 2009). Moreover, in developed countries, strongyloidiasis remains an issue for immigrants (Gonzalez et al. 2010; Sudarshi et al. 2003), tourists (Angheben et al. 2011) and military (Gill et al. 2004) returning from deployment in endemic areas. This fact has implications for medical services in developed countries, and may call for systematic screening after visits to endemic countries and before initiation of immuno-suppressive treatment.

While information on *S. stercoralis* infection rate is patchy, information on incidence is virtually non-existent. None of the identified studies offered evidence on first or new infections. Incidence rates would give insight into how often and how quickly people are re-infected after successful treatment. Further, it could establish how often first-time infections are sustained over a longer period. We showed that prevalence rates in children are often lower than in adults, yet the incidence might be a lot higher if in fact many adult patients acquired the infection during childhood. In addition, risk for infection might be different in children than in adults. Longitudinal studies, particularly at community level, are required to address this knowledge gap.

Comparing the infection rates from hospitalized patients and infection rates in the communities in the same countries often shows great differences. Venezuela and Zambia are good examples, reporting infection rates of 48.4% and 50.6% in hospitalized persons,

respectively; yet in the communities the reported infection rates are as low as 2.3% and 6.6%, respectively. One reason for this discrepancy comes from the use of low-sensitivity methods in community-based studies versus use of moderate- and high-sensitivity methods in the hospitals. Furthermore, hospitalized persons are more likely to belong to an at-risk group or have underlying risk factors for infection with *S. stercoralis*. Additionally, in the hospitals, patients are sampled for more than one day. Another factor is the small number of studies contributing to the calculation of the infection rates. For countries with many studies available (most notably Brazil and Thailand), the differences between the infection rates in communities and in hospitals are considerably smaller (Brazil 13.0% vs. 17.0% and Thailand 23.7% vs. 34.7%). These findings imply that countries with few community-level studies that report high infection rates in the hospitals are likely to be highly endemic. Examples might include DR Congo and Madagascar, both of which lack studies undertaken at community-level yet report infection rates of 32.7% and 52.2% in hospitalized persons, respectively. Here, cross-sectional studies at community level that apply high-sensitivity diagnostic methods and that preferably investigate several stool samples per person over consecutive days are desperately needed to identify possible hotspots of *S. stercoralis* transmission and to quantify the infection rates and risks.

With our approach, we can for the first time report country-wide infection rates. Yet, sometimes a large part of the studies were conducted in a comparatively small area in a specific country. This presents a limitation to our analysis, as do countries with only one or a few studies from a specific location, as it is not possible to make a general statement about prevalence that encompasses all parts of the country. It is very likely that the studies were conducted in areas where *S. stercoralis* infection was already suspected. This is especially true for bigger countries that often have a wide variety of ecological and economic environments, different standards of sanitation, and big differences between rural and urban environments.

A major challenge of giving an overview of prevalence data for *S. stercoralis* world-wide lies in the low comparability of the studies reporting infection rates. Most studies that we identified did not focus on *S. stercoralis* specifically, but on other STHs. Therefore, *S. stercoralis* is mostly reported as an additional outcome and the diagnostic methods used possess only a low sensitivity for *S. stercoralis*. Direct smears and the Kato-Katz method were most commonly used, both of which show a very low sensitivity for the diagnosis of *S. stercoralis* (Sato et al. 1995b; Siddiqui and Berk 2001; Steinmann et al. 2007). The more sensitive and *Strongyloides* specific methods, such as the Baermann method and Koga Agar plate culture are more cumbersome and/or time- and resource intensive (Agrawal et al. 2009). In our model for estimating country-wide infection rates, we addressed this limitation

by taking into account the sensitivity of the diagnostic methods used, summarized as a range derived from the literature.

To further increase diagnostic sensitivity, more than one stool sample should be examined from the same individual over consecutive days (Dreyer et al. 1996; Knopp et al. 2008; Marti and Koella 1993; Satoh and Kokaze 2004). This is also true for superior methods like Baermann or Koga Agar plate culture (Khieu et al. 2013a; Schär et al. 2013a). This is necessary because of the irregular excretion pattern of *S. stercoralis* larvae. Especially for low-intensity infections, there is a big risk that a one-day examination will miss the infection altogether. However, in most studies, only one stool sample was examined. Therefore, the reported infection rates are very likely underestimations.

The challenges outlined above lead to a very heterogeneous set of prevalence data. Today, many countries (including some of the most populous ones) with ecologically and socio-economic conditions favorable to *S. stercoralis* transmission are lacking prevalence data entirely. More data is required for almost all countries and for various socio-economic/cultural settings. Further large-scale surveys that sample the general population, and use highly sensitive methods over three consecutive days would help to narrow this gap.

Finally, as comprehensive as the collection of information on global *S. stercoralis* infection rates was, important information might have been missed due to language restrictions and the choice of databases searched.

5.5.2 Risk groups for *S. stercoralis* infection

Several possible risk factors for *S. stercoralis* infection are reported in the literature. However, studies that focus specifically on risk groups are very rare. We conducted a meta-analysis of case-control studies that provided information on risk and control groups. Most studies were related to HIV/AIDS infection. Our analysis showed an *S. stercoralis* infection risk for HIV/AIDS patients that was twice as high as the risk for individuals without HIV/AIDS (OR: 2.17, 95% BCI: 1.18-4.01). Most studies used the same diagnostic methods for cases and controls, yet the study of Feitosa and colleagues (Feitosa et al. 2001) used additional high sensitivity methods in the HIV-positive group. Another significant highly increased risk for *S. stercoralis* infection was alcoholism (OR: 6.69, 95% BCI: 1.47-33.8). The well-established risk factors HTLV-1 infection as well as diarrhea both showed an increased risk, but without statistical significance (OR: 2.48, 95% BCI: 0.70-9.03 and OR: 1.82, 95% BCI: 0.19-12.2, respectively).

Cases for which strongyloidiasis would cause severe complications in HIV-infected persons are rare. As Keiser & Nutman (Keiser and Nutman 2004) pointed out, less than 30 cases of hyperinfection in HIV-infected individuals have been reported in the literature thus far. The modulation of the immune system by the HIV appears to be the main reason for this. The increase of TH2 cytokines and the decrease of TH1 cytokines (Barker et al. 1995; Tanaka et al. 1999; Valdez and Lederman 1997) leads to a pattern that may favor bacterial and viral opportunistic infections rather than helminthic infections (Concha et al. 2005). Further, it has been proposed that indirect larval development is promoted in patients that are immuno-compromised by advancing AIDS and therefore, the possibility of increased auto-infection is reduced (Viney et al. 2004).

All case-control studies included in the meta-analysis for HTLV-1 (Chieffi et al. 2000; Courouble et al. 2004; Einsiedel and Woodman 2010; Nera et al. 1989) showed an increased risk for *S. stercoralis* co-infection for individuals with an HTLV-1 infection. The result of the meta-analysis however showed no statistically significant risk increase in HTLV-1 infected individuals. As there were only four studies that could be included in the meta-analysis, which is a possible limitation, further case-control studies would be needed to come to a unifying conclusion.

Alcohol-addiction is another potential risk factor for *S. stercoralis* infection. Studies undertaken in Brazil (de Oliveira et al. 2002; Marques et al. 2010; Zago-Gomes et al. 2002) showed evidence of this. It is argued that the regular ethanol intake modulates immune response, making survival and reproduction of the larvae in the duodenum easier. Consequently, there is a higher frequency of larvae present in the stools of alcoholic patients, yet an increased infection rate is not necessarily observed.

For patients with malignancies and/or immuno-compromising conditions, case-control studies are also scarce. De Paula and colleagues (de Paula et al. 2000) showed a higher prevalence of *S. stercoralis* in immuno-compromised children compared to immuno-competent children, although these differences could only be shown with serological diagnostic methods. Using coprological methods, there was no difference in prevalence found between the two groups. This might be because serological diagnostic methods are known to cross-react with other helminth infections or because of the higher sensitivity. Three other case control studies showed a higher prevalence in patients with malignant diseases or undergoing immuno-supressive treatment (Abaza et al. 1995; Hirata et al. 2007; Khalil et al. 1991).

Age-related findings suggest that children are not generally at a higher risk for *S. stercoralis* infection. However, behavioral factors might increase the risk of infection, and many of the infected adults might have picked up an infection during childhood and sustained it through

auto-infection. The infection rates in children lower than or equal to those in adults suggests that due to the persistence of *S. stercoralis*, infections are accumulated over time. Longitudinal studies are needed to get more insight into the incidence and possible accumulation, following the same individuals over longer time periods.

Discerning the risk factors or possible risk factors for *S. stercoralis* infection is hindered by the small amount of research on *S. stercoralis* in general. Therefore, for most risk factors, only a few case-control studies exist, making it difficult to present clear statements. However, these studies can point to trends and lead the way for further and more detailed research.

5.5.3 Diagnostic test sensitivity

Diagnostic tests with low or moderate sensitivity underestimate disease prevalence. The inclusion of the diagnostic test sensitivity in the models allowed us to properly evaluate prevalence and OR for the risk factors under study. The sensitivity adjusted OR for each risk factor have larger uncertainty (wider BCI) most likely due to the added variability of the detection. Furthermore, the intensity of infection influences the sensitivity estimates (Siddiqui and Berk 2001). Higher sensitivity estimates in hospital based surveys may reflect high intensity probably due to co-infection. Test-specific diagnostic sensitivity could not be obtained because of the variety of tests employed in the studies reviewed and relatively small sample size for each test.

5.5.4 What should be done next?

We showed that in many countries, prevalence of *S. stercoralis* infection is high. The results are based on studies that often do not focus on *S. stercoralis* specifically, but on other STHs. Therefore, the results are mostly based on low-sensitivity diagnostic methods and likely underestimate prevalence. It is necessary to conduct further studies using high sensitivity diagnostic methods, coprologically the Koga Agar plate culture or the Baermann or the ELISA in serology, to achieve a more comprehensive and detailed picture of the global prevalence of *S. stercoralis*. Especially in countries with favorable conditions for *S. stercoralis* transmission, studies conducted on STHs should not neglect to include *S. stercoralis*. This would help to establish more detailed data on regional and country-wide prevalence rates. The results obtained in these studies and of our analysis show many countries with a high estimation of the prevalence rate of *S. stercoralis*. In many of these countries the current policy guidelines neglect or are unclear about how to address *S. stercoralis*. We conclude that *S. stercoralis* is of high importance in global helminth control and should therefore not be neglected.

5.6 Supporting information

A PRISMA checklist, PRISMA flow diagram and a web-based reference list, including all studies of the review, can be accessed online

5.7 Acknowledgements

5.8 Author contributions

Conceived and designed the experiments: UT PO. Performed the experiments: FS UT. Analyzed the data: FS FG PV. Contributed reagents/materials/analysis tools: VK SM HM PO. Wrote the paper: FS UT PO. Assisted with manuscript revisions: FG PV VK HM SM. Supervised the first author in all aspects of the review: PO.

6. Treatment of *Strongyloides stercoralis*

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Working paper

6.1 Introduction

The soil-transmitted nematode *Strongyloides stercoralis*, which occurs world-wide, is the most neglected helminth of all neglected tropical diseases (NTDs) and today, still little is known about the global prevalence (Bethony et al. 2006; Genta 1989; Schär et al. 2013b). Three species of *Strongyloides*, which is classified in the order *Rhabditoidea*, infect humans, namely *S. stercoralis, S. fulleborni* and *S. cf fulleborni*, with *S. stercoralis* being by far the most frequent (Genta 1989; Grove 1996; Liu and Weller 1993). After first detection of *S. stercoralis* in 1876, the full life-cycle, clinical features and pathology in man were disclosed in the 1930ies. Infestation can cause a systemic infection of the host's organism. The migrational route of *S. stercoralis* through the human body leads to a constant exposure of a large number of tissues with one or more forms of the parasite. Contingent upon the degree of clinical disease, which the parasite does elicit in the individual host, pathological changes can be observed in a wide range of organs.

S. stercoralis has the unique ability to establish a cycle of repeated endogenous autoinfection within the same host. It therefore can maintain long-term survival, up to 65 years within the same infected individual (Concha et al. 2005; Genta 1992; Keiser and Nutman 2004; Vadlamudi et al. 2006).

Due to its ability of autoinfection and the very rare occurrence of spontaneous cure total extinction of the parasite from the individual host organism must be the goal of treatment. In contrast to many helminth infections a simple reduction of worm burden in the individual below the level at which clinical disease occurs (Grove 1996; Liu and Weller 1993) is not an appropriate approach since this cannot definitely exclude the risk of hyperinfection syndrome or even disseminated Strongyloidiasis and the high mortality rates related to it. Thus, an efficacious drug should ideally eradicate all female worms and autoinfective larvae at large. In reality this is not at least hindered by the relative resistance of the autoinfective larva to chemical agents (Grove 1996; Liu and Weller 1993).

Vaccines against helminths are at best at very early stages of development and do not yet constitute an alternative approach to control infection in humans nor animals (Maizels et al. 1999).

Due to the lack of highly sensitive and specific diagnostic techniques an accurate efficacy evaluation of specific drugs in clinical trials remains a challenge. In this context, some investigators have pointed to the necessity of only enrolling those patients into clinical trials of drug efficacy who presented at least two larvae-positive stool samples out of four consecutives examined (Dreyer et al. 1996). This takes the known irregular excretion rate of

larvae in stool samples into account, particularly among asymptomatic patients with chronic infection. Alternatively, a diagnostic antibody titre of 1:80 or higher in ELISA testing has been proposed as a reliable cut-off for inclusion into drug trials and serological assessment of efficacy (Boscolo et al. 2007).

There is evidence that male patients are more resistant to treatment than females (Satoh et al. 2004; Toma et al. 2000). Patients with proven HTLV-1 infection are reported to be more frequently *Strongyloides* co-infected and moreover, are exposed to a higher risk to develop severe strongyloidiasis (Gotuzzo et al. 1999; Terashima et al. 1999). Additionally, physicians often face significant difficulties to eradicate the Nematode effectively in HTLV-1 infected individuals (Sato et al. 1994; Shikiya et al. 1992; Terashima et al. 2002).

We tried to assess the drugs available today for the treatment of *S. stercoralis* and summarize their efficacy with the help of meta-analysis.

6.2 Methods

6.2.1 Search strategy and selection criteria

A systematic literature review has been conducted. All research papers which have been published until July 2013 in PubMed were screened by using the search terms "*Strongyloides*" OR "*Strongyloides stercoralis*" OR "*Strongyloidiasis*" with "Treatment" OR "Ivermectin" OR "Thiabendazole" OR "Albendazole" OR "Mebendazole" OR "Tribendimidine" OR "Pyrvinium Pamoate". Title and abstract of all identified studies were viewed. Studies were included if they contained information on: treatment regimen and numbers of treated individuals

Excluded were articles (i) not written in English, Spanish, Portuguese, French and German language; (ii) not stating diagnostic methods applied, on infection in animals and (iv) case reports that did not provide additional information on the above mentioned aspects.

6.2.2 Statistical analysis

The meta-analysis of treatment cure rates was described by a Bayesian hierarchical model to take into account the heterogeneity arising from the different regimens and diagnostic tools used in the studies. In each study, the number of people found positive for *S. stercoralis* N_{ij}^+ (out of the total N_{ij} screened) was observed again after administrating treatment i with

regimen j where $i = 1,2,...,T$ and $j = 1,...,r_i$. Overall, 14 different treatments were found (T=14) and the number of regimens r_i depended on the treatment i. The same diagnostic tool was used in the two screening rounds of a study, but different tools were used across studies. Diagnostic tools were aggregated into $k = 3$ groups according to their sensitivity s and classified as low, medium and high, following information found in the literature. This approach was previously used by the authors (Schär et al. 2013b). The number of individuals with cleared *S. stercoralis* infection (i.e. cured) after treatment, denoted as Y_{ij}, follows a Binomial distribution $Y_{ij} \sim (N_{ij}^+, CR_{ij}(s_k))$ where CR_{ij} is the observed cure rate for treatment i and regimen j that used a diagnostic tool with sensitivity of category k. The true cure rate CR_{ij} is related to the observed cure rate via the equation $CR_{ij}(s_k) = CR_{ij}s_k$. To estimate the overall cure rate for treatment $i, (CR_i)$ a logistic transformation was applied, i.e. $\text{logit}(CR_{ij}) = \text{logit}(\text{CR}_i) + \delta_j$ where δ_j models the variability across regimens within the same treatment scheme. Normal prior distribution were assumed for δ_j: $\delta_j \sim N(0, \sigma_i^2)$ where σ_i^2 is the variance associated to treatment i. Prior distributions assigned to the sensitivity categories were Beta distributions with parameters defined in a previous study (Schär et al. 2013b). Studies that did not report data on the diagnostic tool used to perform estimation of the treatment performance were assigned a uniform prior distribution on the sensitivity (non-informative).

The model was fitted in WinBUGS4 (Lunn et al. 2000) and the plots were produced in R version 3.0.3. The results are reported in terms of estimated median cure rates and corresponding Bayesian confidence intervals (95% BCI).

6.3 Results

The literature identified 14 drugs that were used for treatment of *S. stercoralis,* with four applied in more than 3 studies (Figure 6.1). In total, we included 68 studies. Ivermectin featured in 30 (44.7%), Albendazole in 23 (33.8%), Thiabendazole in 21 (30.9%) and Mebendazole in 5 (7.4%) of the studies, respectively:

6.3.1 Ivermectin

Ivermectin, a macrocyclic lactone, was found to be an anthelminthic substance in 1975 and commercially introduced in 1981 (Campbell 2012; Campbell et al. 1983; Chabala et al. 1980). It is part of the Avermectin family, derived from the actinomycete *Streptomyces*

avermectinius (Molinari et al. 2010). It causes paralysis of nematodes through the influx of chloride ions across cell membranes (Ottesen and Campbell 1994). It has a half life of 18 hours and is mainly excreted by faeces. The most commonly reported side-effects are mild, including headache, dizziness, muscle pain, nausea or diarrhea. It is administered orally, and because of the comparably small amount of drug necessary, the Ivermectin tablets are small and easy to swallow. This is an advantage when treating children. In several case-reports regarding to the efficacy and safety a parenteral or subcutaneous administration of a veterinary formulation of Ivermectin was administered. This therapy was used in clinical situations which were difficult to master using oral substances (Chiodini et al. 2000; Marty et al. 2005; Pacanowski et al. 2005; Salluh et al. 2005; Turner et al. 2005).

Several studies have proven the superiority of Ivermectin over alternative drugs in terms of efficacy and side effects (Adenusi 2003; Behnke et al. 1994; Caumes et al. 1993; Datry et al. 1994; Gann et al. 1994; Heukelbach et al. 2004a; Heukelbach et al. 2004b; Igual-Adell et al. 2004; Lindo et al. 1996; Marti et al. 1996; Naquira et al. 1989; Ordonez and Angulo 2004; Shikiya et al. 1991; Shikiya et al. 1994; Toma et al. 2000; Zaha et al. 2000; Zaha et al. 2002).

We could identify a total of 40 different studies/regimens applied for the treatment of *S: stercoralis*, treating a total of 1778 individuals. The meta-analysis revealed a very good cure rate of 0.97 (95% BCI: 0.94 – 1.00). This is the best cure rate we observed in our analysis (Figure 2).The regimen most commonly applied was the standard dose of 200µg/kg as a single dose. Interestingly, the cure rate observed for this standard treatment was the lowest observed, although with 0.95 (95% BCI: 0.92 – 1.00) it is only marginally lower than the pooled cure rate. The best cure rate observed was the standard treatment applied on two days. This increased the cure rate to 0.98 (95% BCI: 0.96 – 1.00). Seven studies reported in detail on adverse events. The most commonly observed adverse events were dizziness and nausea, both reported in five studies and fatigue in four studies. They were only detected in a small number of individuals, for dizziness in 0.6% up to 4.9%, for nausea in 0.2% to 4.1% and for fatigue in 0.8% up to 13.1%, respectively (Figure 6.2 and Table 6.1).

Ivermectin is considered to be the drug of choice today, even in cases of disseminated infection (Albonico et al. 1999). After worldwide distribution of millions of doses in mass treatment programs against the two systemic filarial nematodes of man, *Onchocerca volvulus* and *Wuchereria bancrofti,* it can be regarded as a fairly safe drug. However, there is restriction of use in several patient sub-groups (children under 5 years of age or weighing less than 15 kg, in pregnancy and lactation, in patients with diseases of the central nervous system) (Fox 2006). However, recently the drug has been administered without causing serious and long-lasting adverse effects in children less than 5 years of age treated for

proven *Strongyloidiasis* (Ordonez and Angulo 2004) and scabies (del Mar Saez-De-Ocariz et al. 2002; Lawrence et al. 2005). In individuals with heavy *Loa Loa* microfilariae infection a severe encephalopathy has been reported with Ivermectin (Gardon et al. 1997).

Summarized it is save to say that Ivermectin is a strong and suitable candidate for the use in mass treatment in populations infected with *S. stercoralis* and other helminths, as well as filarial nematodes and ectoparasites.

| **Treatment for *Strongyloides stercoralis*** | | | | | |
Benzimidazoles	Drug Info (Structure, Systematic name, half-life)	Treatment regimen (recommended for SS)	Possible Side effects	Advantages	Disadvantages
Albendazole	Methyl [6-(propylthio)-1 *H*-benzoimidazol-2-yl]carbamate 8.5 hours	Two courses 400 mg twice daily on three consecutive days	Headache Dizziness Fever Nausea Vomiting	Commonly used for treatment of other helminthic infections	Low efficacy
Mebendazole	Methyl (5-benzoyl-1 *H*-benzimidazol-2-yl)carbamate 2.5 to 5.5 hours	200 mg daily for 28 consecutive days	Diarrhea Abdominal Pain Headache Fever Dizziness Urticaria Liver dysfunction	Commonly used for treatment of Hookworm and *T. trichuris*	Low efficacy especially with reduced treatment regimens
Thiabendazole	4-(1 *H*-1,3-benzodiazol-2-yl)-1,3-thiazole 8 hours	two or three courses of 25-50 mg/kg/day for three consecutive days	General fatigue Dizziness Headache Nausea Appetite Loss Abdominal pain Liver dysfunction Neuropsychiatric symptoms	Very effective	Side effects are very common
Macrocyclic lactones					
Ivermectin	Ivermectin (22,23-dihydroavermactin B1a + 22,23-dihydroavermectin B1b 18 hours	200 µg/kg single dose, on two consecutive days	Loss of appetite Dizziness Nausea Vomiting Stomach pain or bloating Diarrhea Constipation Weakness Sleepiness	Most effective	Expensive
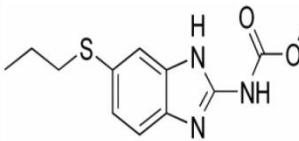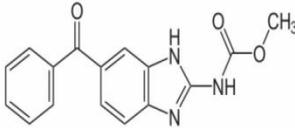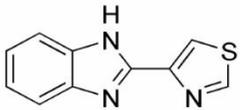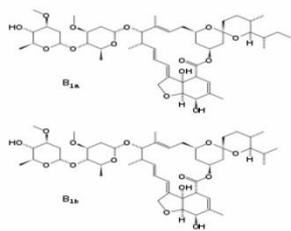
Figure 6.1 – Information on the four most common drugs for *S. stercoralis* treatment

6.3.2 Thiabendazole

Thiabendazole is a benzimidazole that inhibits the mitochondrial helminth-specific enzyme fumarate reductase. It was originally of veterinary use, and allowed for human use in 1967 (Gilman and Goodman 1990). It has a short half-life of 8 hours, excretion is 90% with the urine. Thiabendazole has a high rate of side effects including general fatigue, dizziness, headache, nausea, appetite loss, abdominal pain, liver dysfunction and neuropsychiatric symptoms (Grove 1982).

We could identify 24 studies/regimes, with a total of 1048 individuals treated. The meta-analysis showed a pooled cure rate of 0.95 (95% BCI: 0.92 – 0.99), almost as good as the cure rate of Ivermectin. The most commonly applied regimes were either 25mg/kg for three days, repeated after one week, or 25mg/kg twice for three days; both totalling in a total drug dosage of 150mg/kg. The lowest cure rate (0.91 (95% BCI: 0.84 – 0.98)) was observed for the regimen of 25mg/kg for 2 days. The best cure rate of 0.97 (95% BCI: 0.95 – 0.99) was observed for the regimen of 1500mg for five days, repeated for 3 or 4 courses, totalling in 22.5g and 30g of thiabendazole administered, respectively. Adverse events are rarely reported in detail, yet in one study 49.5% of treated individuals reported fatigue and another 45.0% nausea (Figure 6.2 and Table 6.1).

Thiabendazole showed an excellent efficacy for the treatment of *S. stercoralis*. Yet, the major drawbacks of this substance are the frequent and sometimes severe adverse events. The drug cannot be considered first choice anymore and should only be used if Ivermectin is not available (Zaha et al. 2000).

6.3.3 Mebendazole

Mebendazole is another benzimidazole, as Thiabendazole. It was first synthesized in 1968 (Banerjee et al. 1972). Today, Mebendazole is widely used throughout the world for the treatment of STHs (Keiser and Utzinger 2008; Utzinger and Keiser 2004). It has a half life time of 3-6 hours and is excreted mainly by faeces. It is mainly used to treat *Trichuriasis* and hookworm (Liu and Weller 1996) but can also be administered in the treatment of *S. stercoralis* (Albonico et al. 1999; Zaha et al. 2000). Its mode of action is by selectively inhibiting the synthesis of microtubules in the parasite, blocking the uptake of glucose and other nutrients. This results in the gradual immobilization and death of the parasite. The most common side-effects reported are diarrhea, fever, abdominal pain, heart pain, slight headache, dizziness, exanthema, urticaria, and angioedema.

Treatment	No. of studies	Cured/Treated	Cure rate (95% BCI)
Ivermectin			
IVM1	18	705/826	0.95 (0.92 – 1.00)
IVM2	1	8/12	0.97 (0.91 – 1.00)
IVM3	2	11/13	0.97 (0.92 – 1.00)
IVM4	1	4/5	0.97 (0.92 – 1.00)
IVM5	6	463/491	0.98 (0.95 – 1.00)
IVM6	12	414/431	0.98 (0.96 – 1.00)
Pooled	40		0.97 (0.94 – 1.00)
Thiabendazole			
TBZ1	3*	83/107	0.91 (0.84 – 0.98)
TBZ2	2	103/128	0.92 (0.86 – 0.99)
TBZ3	1	3/6	0.95 (0.90 – 0.99)
TBZ4	5	233/255	0.95 (0.92 – 0.99)
TBZ5	1	12/12	0.96 (0.91 – 0.99)
TBZ6	1*	30/30	0.96 (0.92 – 0.99)
TBZ7	6	156/202	0.96 (0.93 – 0.99)
TBZ8	4	139/146	0.97 (0.95 – 0.99)
TBZ9	1	162/162	0.95 (0.92 – 0.99)
Pooled	24		0.95 (0.92 – 0.99)
Mebendazole			
MBZ1	1	10/28	0.71 (0.56 – 0.83)
MBZ2	1	25/31	0.84 (0.74 – 0.92)
MBZ3	1	27/33	0.85 (0.74 – 0.92)
MBZ4	1	14/21	0.86 (0.73 – 0.93)
MBZ5	1	15/16	0.90 (0.82 – 0.95)
MBZ6	1	47/47	0.84 (0.76 – 0.91)
Pooled	6		0.84 (0.76 – 0.91)
Albendazole			
ABZ1	13	457/664	0.74 (0.70 – 0.78)
ABZ2	1	1/8	0.74 (0.59 – 0.85)
ABZ3	2	27/46	0.77 (0.66 – 0.86)
ABZ4	1	4/9	0.78 (0.64 – 0.87)
ABZ5	1	13/19	0.79 (0.68 – 0.88)
ABZ6	1	2/4	0.80 (0.66 – 0.89)
ABZ7	2	33/47	0.81 (0.71 – 0.89)
ABZ8	1	8/9	0.82 (0.71 – 0.90)
ABZ9	1	21/24	0.83 (0.74 – 0.91)
ABZ10	3	60/75	0.84 (0.77 – 0.91)
ABZ11	2	69/76	0.87 (0.81 – 0.92)
ABZ12	6	152/225	0.88 (0.82 – 0.92)
Pooled	34		0.81 (0.75 – 0.86)

Figure 6.2 – Pooled cure rates estimated by meta-analysis, for the four main drugs
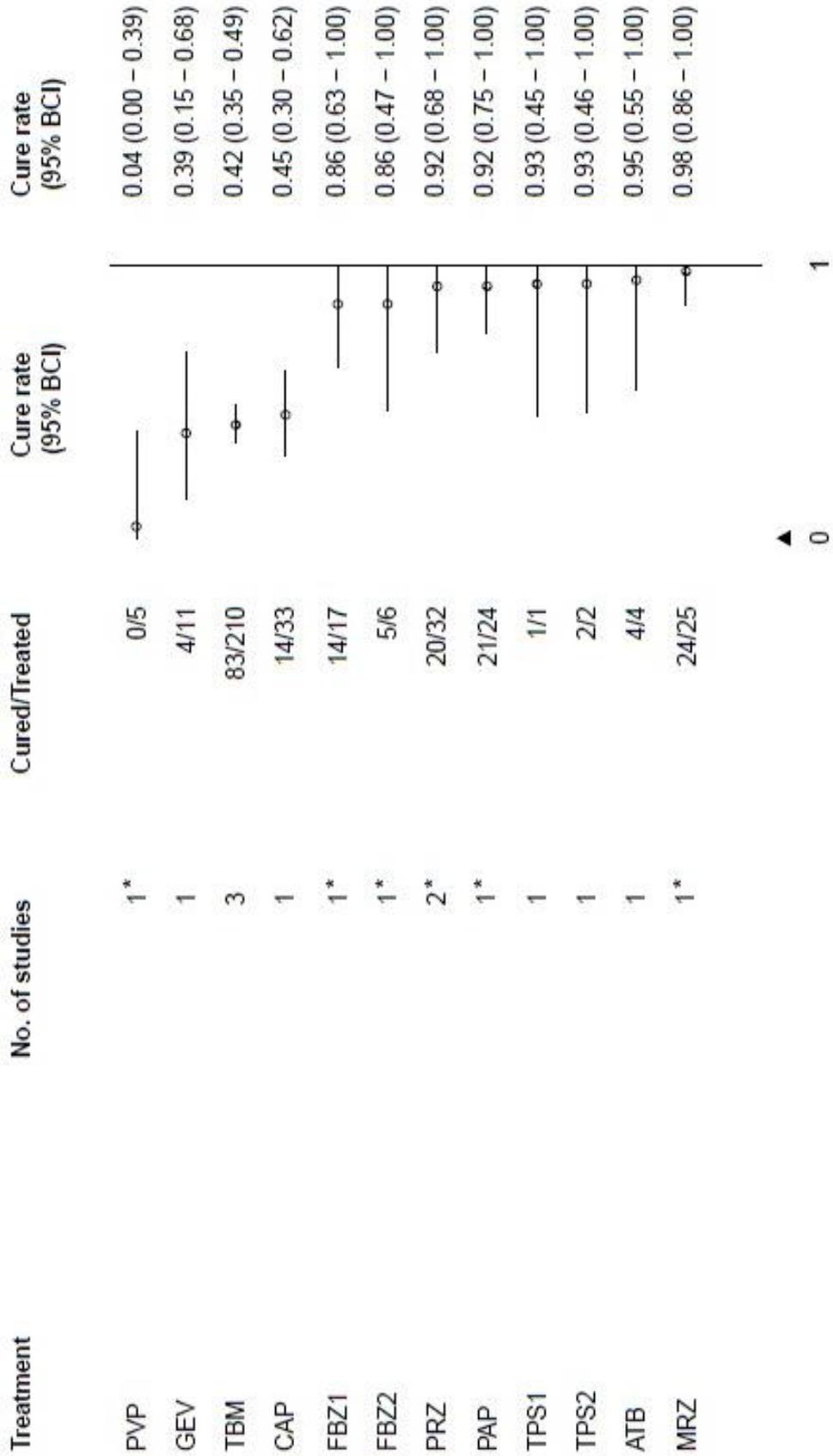
Figure 6.3 - Pooled cure rates estimated by meta-analysis, all other drugs

Table 6.1 – Treatment regimens applied. The abbreviation in the Treatment column refers to the results displayed in Figure 6.2 and Figure 6.3 (continued on next page)

Drug	Treatment	Regimen	Total Drug used
Ivermectin	IVM1	200µg/kg, sd	200µ/kg
	IVM2	50µg/kg, sd	50µg/kg
	IVM3	150µg/kg, sd	150µg/kg
	IVM4	100µg/kg, sd	100µg/kg
	IVM5	6mg, sd, repeated after 2 wks	12mg
	IVM6	200µg/kg, sd, 2 days	400µ/kg
Thiabendazole	TBZ1	25mg/kg, 2 days	50mg/kg
	TBZ2	50mg/kg, twice for 3 days	300mg/kg
	TBZ3	30mg/kg	30mg/kg
	TBZ4	25mg/kg, 3 days, repeated after 2 wks OR 25mg/kg, twice for 3 days	150mg/kg
	TBZ5	1g twice daily, 5 days	10g
	TBZ6	25mg/kg, twice for 5 days	250mg/kg
	TBZ7	25mg/kg, twice for 2 days	100mg/kg
	TBZ8	25mg/kg, twice for 2 days, repeated after one wk	200mg/kg
	TBZ9	1500mg for 5 days, 3-4 courses every 2 wks	30g
Mebendazole	MBZ1	400mg, 3 days	1200mg
	MBZ2	25mg/kg, 3 days	75mg/kg
	MBZ3	200mg for 28days	5600mg
	MBZ4	100mg, twice for 4 days	800mg
	MBZ5	200mg, 4 days, repeated after one wk	1600mg
	MBZ6	200mg, 5 days, repeated 3 wks	3000mg
Albendazole	ABZ1	400mg, 3 days, repeated after 2 wks OR 400mg, twice daily for 3 days	2400mg
	ABZ2	400mg, twice for 3 days, repeated after one wk	4800mg
	ABZ3	400mg, twice for 7 days	5600mg
	ABZ4	4mg/kg, 5 days	20mg/kg

	ABZ5	16mg/kg, 6 days	96mg/kg
	ABZ6	8mg/kg, 3 days	24mg/kg
	ABZ7	400mg, 5 days	2000mg
	ABZ8	16mg/kg, 3 days	48mg/kg
	ABZ9	20mg/kg, 5 days	100mg/kg
	ABZ10	400mg	400mg
	ABZ11	400mg, twice for 5 days	4000mg
	ABZ12	400mg, 3 days OR 200mg, twice for 3 days	1200mg
Pyrvinium Paomate	PVP	5mg/kg, 3 days, repeated after 2 wks	30mg/kg
Gentian Violet	GEV	90mg, 14 days	1260mg
Tribendimidine	TBM	400mg	400mg
Carica papaya	CAP	20ml, sd (equals 4g of C. papaya)	4g
Flubendazole	FBZ1	200mg, 3 days	600mg
	FBZ2	300mg, 3 days	900mg
Piperazine	PRZ	0.6g, five times for 5 days	15g
Pyrantel Paomate	PAP	10mg/kg, 3 days	30mg/kg
Trichlorophenol-Piperazine Salt	TPS1	1.5g, twice for 3 days	9g
	TPS2	3g, twice	6g
Atabrine	ATB	30cc 1% aqueous solution intraduodenally	
Mirazid	MRZ	600mg, repeated weekly for a month	14400mg

We could identify six different studies/regimes, with a total of 176 individuals treated. The pooled cure rate established was 0.84 (95% BCI: 0.76- 0.91). The best treatment regimen was 200mg on 5 days, repeated 3 weeks. This total of 3000mg Mebendazole had a cure rate of 0.90 (95% BCI: 0.82 – 0.95). It was also the treatment regimen applied in most individuals (47/176, 26.7%). By far the worst regimen was 400mg for three days, with a cure rate of 0.71 (95% BCI: 0.56 – 0.83), which was administered in 28 individuals (Figure 6.2 and Table 6.1).

6.3.4 Albendazole

Albendazole is also a benzimidazole. It was first used in humans in 1987 (Ottesen et al. 1999). It has a half life time of 8-12 hours, and is excreted through the urine and faeces. Albendazole, like Mebendazole, causes degenerative alterations in the tegument and intestinal cells of the parasite by binding to the colchicine-sensitive site of tubulin. This inhibits its polymerization or assembly into microtubules. Without the cytoplasmic microtubules the uptake of glucose by the larval and adult stages of the susceptible parasites is impaired, depleting their glycogen stores. Common side-effects include abdominal pain, dizziness, headache, fever, nausea, and vomiting. Less frequent are severe headaches, seizures, vision problems, persistent sore throat and mood changes. There can be an elevation of liver enzymes during treatment, which in rare cases can cause acute liver failure (Marin Zuluaga et al. 2013).

We identified 34 studies/regimens, with a total of 1206 individuals treated. The pooled cure rate was 0.81 (95% BCI: 0.82 – 0.86). The regimen applied by far the most was a total drug amount of 2400mg, either as 400mg on three days, repeated after one week or as 400mg twice per day for 3 days. This regimen showed the lowest cure rate with 0.74 (95% BCI: 0.70 – 0.78). Surprisingly, the best regimen, applied in 225 individuals, was only half the amount of Albendazole administered (1200mg), either as 400mg for three days or 200mg, twice daily for 3 days, showing a cure rate of 0.88 (95% BCI: 0.82 – 0.92). From five studies reporting on the adverse events, the most common one was dizziness, which was observed in three studies (range: 0.9% to 30.3%) (Figure 6.2 and Table 6.1).

We argue, that because of it moderate efficacy against *S. stercoralis*, Albendazole should only be considered those settings where Ivermectin or even Thiabendazole are not available (Nontasut et al. 2005; Satoh and Kokaze 2004; Singthong et al. 2006).

6.3.5 Other drugs used

All drugs we describe in this chapter have been applied and tested on a small scale. Furthermore, for several surveys included, there was no specification given about what diagnostic method was applied for the detection of *S. stercoralis* and, more importantly, for the estimation of the cure rate. The meta-analysis used an uninformed prior for the sensitivity. The results therefore have to be interpreted with keeping this information in mind. Altogether five drugs were evaluated without information of the sensitivity of the diagnostic methods applied, namely Pyrvinium Paomate, Flubendazole, Piperazine, Pyrantel Paomate and Mirazid. The meta-analysis showed the best cure rate for Mirazid with 0.98 (95% BCI: 0.86 – 1.00). This promising result has to be taken with caution, as there is no information

available on how the treatment success has been evaluated. Another five drugs were evaluated including the sensitivity of diagnostic method. These are Gentian Violet, Tribendimidine, *Carica papaya* (dried papaya seeds), Trichloriphenol-Piperazine Salt and Atabrine. The two best cure rates were observed for Atabrine (0.95, 95% BCI: 0.55 – 1.00) and Trichlorophenol-Piperazine Salt (0.93, 95% BCI: 0.46 – 1.00), respectively. Both drugs were evaluated on a very small number of individuals, hence the large confidence intervals. Atabrine was tested on four individuals, the Trichlorophenol-Piperazine Salt on two and one individual (two different regimens), respectively. It therefore is difficult to draw any firm conclusions on how effective these two treatments are. All other drugs with sensitivity stated achieved a cure rate that was lower than 0.50 (Figure 6.3). This is also true for Tribendimidine, a drug that also has a wide spectrum against helminthic infections (Xiao et al. 2005). Its activity against *Strongyloides* has been documented in the laboratory in in vitro and in vivo studies on *S. ratti* (Keiser et al. 2008). First studies have been conducted in humans in China, with an observed cure rate of 54.5% (Steinmann et al. 2008).

6.4 Discussion

The main challenge for treatment of *S. stercoralis* infections is that unlike with other helminths, total eradication from the individual host organism should be the target (Grove 1996; Liu and Weller 1993). Lowering the worm burden to levels where no clinical symptoms occur does not exclude the risk for hyperinfection syndrome or disseminated strongyloidiasis at a later stage. An efficacious drug should therefore be able to eradicate all female worms and auto-infective larvae.

Assessing the efficacy of the drugs available for treatment is difficult as it is linked with the difficulties in diagnosing *S. stercoralis*. Treated individuals should be followed up for several days to ensure that the infection has been entirely eradicated. This takes the irregular shedding pattern and the difficulties in diagnosis into account, especially amongst asymptomatic patients with a chronic infection.

General observations made about the treatment of *S. stercoralis* include the evidence that male patients are more resistant to treatment than females (Satoh and Kokaze 2004; Toma et al. 2000). Patients with a proven HTLV-1 infection have not only been shown to have a higher risk of developing severe strongyloidiasis (Gotuzzo et al. 1999; Terashima et al. 1999), but they also tend to pose more difficulties for a complete eradication of *S. stercoralis* (Sato et al. 1994; Shikiya et al. 1992; Terashima et al. 2002).

Vaccinations against helminths are only at very early stages and do not yet constitute an alternative approach to control infection in humans (Maizels et al. 1999).

Ivermectin has showed a high efficacy for the treatment of *S. stercoralis*. At current, Ivermectin is in many countries and by WHO not considered as the standard treatment for *S. stercoralis*. Ivermectin is used in combating *Filariasis* and *Onchocerciasis*.

In countries with high prevalence for soil-transmitted helminths, periodical mass drug administration (MDA) is in place, targeted at potential risk groups like children or women in reproductive age. We argue that Ivermectin should be included in the MDA in countries with high STH-prevalence, even if accurate estimates of the prevalence of *S. stercoralis* are missing. There are mainly two reasons which call for the inclusion of Ivermectin in the national MDAs:

1. In countries with a high prevalence of STHs, determined by appropriate diagnostics with a high sensitivity, one can assume a high prevalence for *S. stercoralis* also, especially if the common diagnostic methods could identify a high hookworm prevalence, as these two parasites have a very similar route of transmission.
2. Ivermectin has proven its applicability and safety, as it is used in mass treatments for *Filariasis* and *Onchocerciasis*.

Despite these two points mentioned, the long-term strategy should still include large-scale studies which not only focus on the better known STHs but specifically on *S. stercoralis*. This would enable the responsible authorities of each country to make a final decision about a possible inclusion of Ivermectin in their MDA programmes.

Furthermore there should be additional studies on the safety of Ivermectin in specific risk groups, namely pregnant woman and children younger than five years of age.

In industrialised countries, the main focus for the public health efforts should aim at specific risk groups such as immunocompromised individuals, immigrants and refugees, travellers, military personnel and institutionalised individuals. It is important that appropriate measures concerning the screening methods and the treatment are taken. This helps to reduce the burden on the national health systems and can help to avoid the costly treatment of the potentially life-threatening complications that can occur with an infection with *S. stercoralis*.

The meta-analysis of all the drugs that were applied showed the trends that are known today. The two major antihelminthic drugs, Albendazole and Mebendazole both show an effect against *S. stercoralis*. Yet they are both not satisfyingly effective, especially because for

S. stercoralis the treatment target should be the total eradication of the parasite from the host. Because of the possibility for an auto-infection, only reducing the worm-burden to a level, where no symptoms occur (like with other STH infections) is not acceptable. Therefore, Thiabendazole and Ivermectin are the two drugs to us for treatment. Because of the severe side-effects of Thiabendazole, Ivermectin should always be the drug of choice today.

From the other drugs possible candidates need further evaluation. All of them have been tested in very small samples. Larger-scale examinations, with the use of proper high sensitivity diagnostic methods could help to decide whether one drug should be evaluated further or not.

7. The prevalence and diversity of intestinal parasitic infections in humans and domestic animals in a rural Cambodian village

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7.1 Abstract

In Cambodia, intestinal parasitic infections are prevalent in humans and particularly in children. Yet, information on potentially zoonotic parasites in animal reservoir hosts is lacking. In May 2012, faecal samples from 218 humans, 94 dogs and 76 pigs were collected from 67 households in Dong village, Preah Vihear province, Cambodia. Faecal samples were examined microscopically using sodium nitrate and zinc sulphate flotation methods, the Baermann method, Koga Agar plate culture, formalin-ether concentration technique and the Kato Katz technique. PCR was used to confirm hookworm, *Ascaris* spp., *Giardia* spp. and *Blastocystis* spp. Major gastrointestinal parasitic infections found in humans included hookworms (63.3%), *Entamoeba* spp. (27.1%) and *Strongyloides stercoralis* (24.3%). In dogs, hookworm (80.8%), *Spirometra* spp. (21.3%) and *Strongyloides* spp. (14.9%) were most commonly detected and in pigs *Isospora suis* (75.0%), *Oesophagostomum* spp. (73.7%) and *Entamoeba* spp. (31.6%) were found. Eleven parasite species were detected in dogs (eight helminths and three protozoa), seven of which have zoonotic potential, including hookworm, *Strongyloides* spp., *Trichuris* spp., *Toxocara canis*, *Echinostoma* spp., *Giardia duodenalis* and *Entamoeba* spp. Five of the parasite species detected in pigs also have zoonotic potential, including *Ascaris* spp., *Trichuris* spp., *Capillaria* spp., *Balantidium coli* and *Entamoeba* spp. Further molecular epidemiological studies will aid characterisation of parasite species and genotypes and allow further insight into the potential for zoonotic cross transmission of parasites in this community.

Key Words: dogs, pigs, gastrointestinal parasites, zoonosis, Cambodia

Highlights

Multi-parasitism is common in humans, dogs and pigs in rural Cambodia

Dogs and pigs are potential reservoirs for zoonotic transmission

Behavioural factors and faeces use by humans show potential risk for zoonotic cross transmission

Graphical abstract
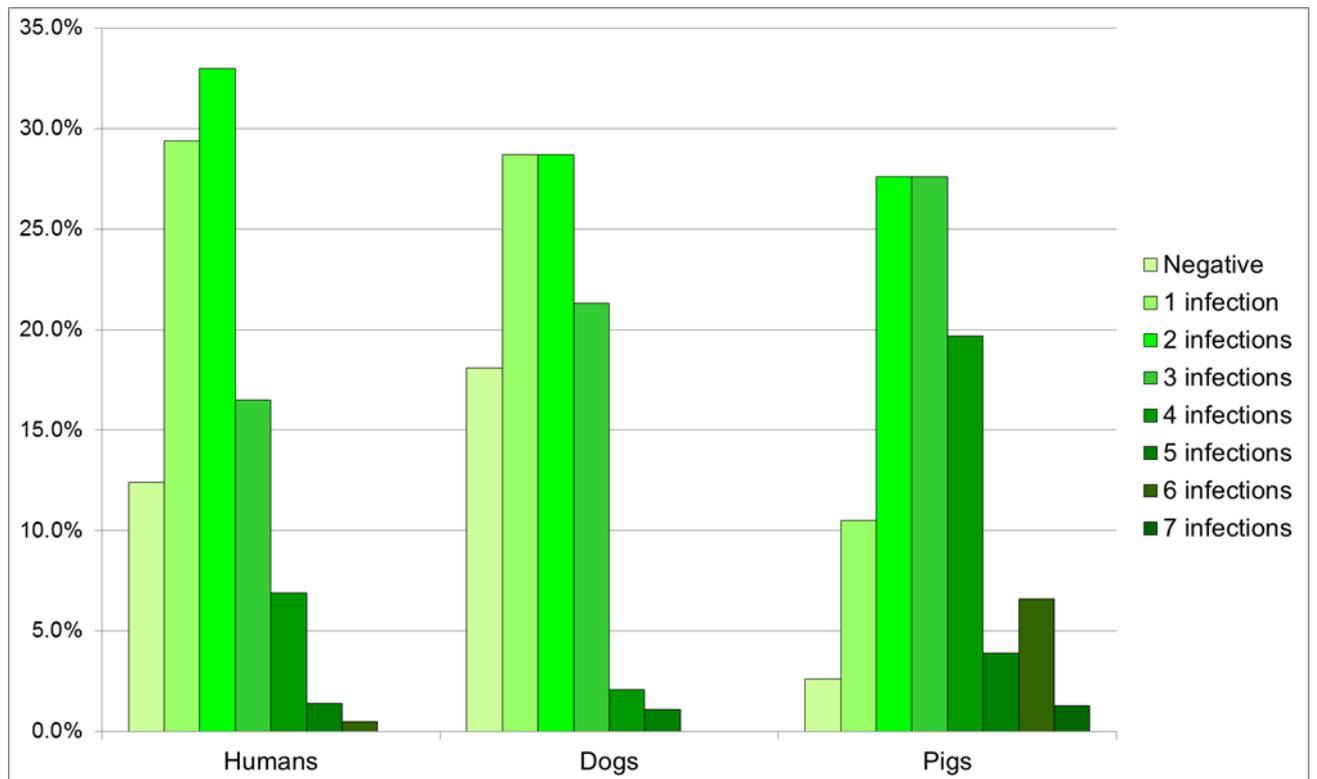
7.2 Introduction

Intestinal parasitic infections (IPIs) are the most common infections among humans and domestic animals such as dogs, cats and pigs, particularly in the rural areas of Southeast Asia. Chronic infections with one or several of the most common soil-transmitted helminths (STHs), *Ascaris lumbricoides*, *Trichuris trichiura* and hookworms, might account for a global burden of 39 million disability-adjusted life years lost annually (Murray et al. 2012). Another STH, *Strongyloides stercoralis,* is often neglected in helminth surveys (Schär et al. 2014b; Schär et al. 2013b), yet previous studies show high *Strongyloides stercoralis* infection rates in Cambodia (Khieu et al. 2013a). School-aged children in the developing world are at highest risk of morbidity due to soil-transmitted helminthiasis and intestinal protozoan infections (Speich et al. 2010).

Many of the IPIs in animals, especially those with the larval stages of hookworms, *Gnathostoma* spp. and *Toxocara* spp., may result in zoonotic diseases such as eosinophilic enteritis (Prociv and Croese 1990), cutaneous larval migrans, and toxocariasis. In addition, dogs and pigs may also serve as definitive reservoir hosts for adult zoonotic parasites capable of forming patent infections in humans, including *Ancylostoma ceylanicum* (dogs) (Areekul 1979), *Ascaris* spp. (pigs) (Zhou et al. 2012), *Trichuris* spp. (pigs) (Nissen et al. 2012), *Fasciolopsis buski* (pigs) (Graczyk et al. 2001)*, Echinostoma* spp. (dogs/pigs) (Lan-Anh et al. 2009)*, Cryptosporidium* spp. (dogs) (Thompson and Smith 2011) and *Giardia duodenalis* (dogs) (Traub et al. 2004a). Current information on the prevalence of such parasites is vital for developing veterinary and public health strategies for their treatment and control (Palmer et al. 2008).

Today, the national helminth control programme covers most school-aged children in Cambodia. However, mass treatment only focuses on three major soil-transmitted helminths (*Ascaris*/hookworm/*Trichuris*). Other nematodes like *S. stercoralis*, trematodes and protozoan infections are neglected.

In rural Southeast Asia, little is known about the zoonotic potential of IPIs in humans and animals. Previous studies have shown a high prevalence of intestinal protozoan and helminthic parasites infecting Cambodian refugees and school children (Gyorkos et al. 1992; Khieu et al. 2013a; Lee et al. 2002; Lurio et al. 1991; Nwanyanwu et al. 1989; Park et al. 2004; Sinuon et al. 2003). The role of domestic animals, such as dogs and pigs, as contributors to human IPI and as reservoir hosts for zoonotic parasites remains unexplored and/or the data are inaccessible. The current study was conducted to obtain information on the prevalence and diversity of IPIs among humans and domestic animals living in the same household in a rural Cambodian village.

7.3 Materials and Methods

7.3.1 Ethical considerations

The study protocol was approved by the Ethics Committee of the Canton of Basel, Switzerland and the National Ethics Committee for Health Research, Ministry of Health in Cambodia. All participants and relevant parties were informed of the purpose of the study. Written informed consent was obtained from all individuals prior to enrolment. All infections diagnosed in humans and animals were treated at the end of the study according to the Cambodian treatment guidelines.

7.3.2 Study design and area

The study was carried out in May 2012 in Dong village, Rovieng district, Preah Vihear province, Cambodia. Preah Vihear province is located in the north of Cambodia, bordering Thailand and Lao PDR (13º47'N 104º58'E). The climate is tropical, with warm and hot temperatures all year round and alternating dry and wet seasons. Households from Dong village were randomly selected from lists provided by the Ministry of Health. All household members (>2 years) and animals (dogs, pigs) were assessed for IPIs using a multiple diagnostic test approach on two stool samples for each human and one sample for each animal. Only animals owned by the household were included in the survey. Risk factors for infection of humans and animals were assessed based on information collected through questionnaire interviews and observations. The questionnaire mainly covered demographic information, occupation, eating habits, personal hygiene practices and household assets. Information about the animals (i.e. age) was collected by questioning the respective owner.

7.3.3 Field procedures and sample collection

On the day of the first visit, informed consent was obtained from all household members and questionnaire-based interviews were conducted with enrolled participants. Interviews with young children were conducted with the help of a parent or legal guardian. The questionnaire covered the following topics: demographics, self-reported symptoms, personal hygiene practices, household assets and food consumption. All enrolled participants received a pre-labelled stool container. Participants were asked to fill the container with faeces passed the following morning. Upon collecting the first sample, a second stool container was given to participants for filling. The collected stool samples were transported within two hours following defecation to a laboratory in Rovieng Health Centre. Stool samples from each dog and pig present at the time of the visit and belonging to the household were obtained. For each animal, approximately five grams of faeces were collected from the rectum directly, placed into a sterile plastic faecal container and chilled immediately in a box containing ice.

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For each human, two stool samples given on consecutive days were analysed and for each animal, one sample was analysed.

7.3.4 Laboratory procedures

For each human stool sample, the following tests were performed: Kato Katz (Katz et al. 1972), Koga Agar culture (Koga et al. 1991), Baermann (Baermann 1917), formalin-ether concentration technique (FECT) (Marti and Escher 1990) and sodium nitrate and zinc sulfate flotation (Inpankaew et al. 2007) analysis. As they arrived in the laboratory, human samples were processed as follows:

First, duplicate Kato Katz smears were prepared. Stool was filtered using a nylon mesh and then placed on the standard Kato Katz template, leaving 41.7 mg of stool for examination on a microscopic slide. Examination was performed at 100x magnification (Katz et al. 1972) for hookworm, *Taenia* spp. and small and large trematode eggs (*Ophisthorchis*/*Chlonorchis*/*Dicrocoelium*-like and *Fasciola*/*Fasciolopsis*/*Echinostomes*). Second, a Koga Agar test was prepared by placing a piece of stool (3–5g) on a freshly produced Agar plate. The plates were then incubated for 48 hours at 28°C. Larvae were washed from the plate into a tube, the liquid was centrifuged and the entire sediment was read at 40x magnification (Koga et al. 1991) for hookworm and *S. stercoralis* larvae. Third, a Baermann test was prepared. In brief, stool was placed in a wire-mesh situated in a funnel. A clamped tube was attached to the funnel while the funnel and tube were filled with tap water. Artificial light was used to stimulate larvae movement out of the stool and into the tube. After two hours, the tube-water was centrifuged. The entire sediment was read at 40x magnification (Baermann 1917) for *S. stercoralis* larvae. Fourth, stool samples were fixed in a 15 ml centrifuge tube with 10 ml of sodium-acetate-formaldehyde (SAF) solution and subsequently analysed for protozoa (*Entamoeba* spp., *G. duodenalis*, *Blastocystis*, *E. nana*, *Isospora* spp. and *I. bütschlii*) at the National Malaria Centre in Phnom Penh, Cambodia, using the FECT method (Marti and Escher 1990). Lastly, two grams of stool were fixed separately in 10% formaldehyde and subjected to sodium nitrate and zinc sulphate flotation (Inpankaew et al. 2007) (Faust et al., 1938) at the School of Veterinary Science, University of Queensland, Gatton campus, Australia.

For the animal samples, a sodium nitrate and zinc sulphate flotation analysis was performed in the same manner as for the human samples (Inpankaew et al. 2007).

Additionally, all human and animal samples were fixed in 2.5% potassium dichromate for subsequent PCR analysis, performed according to the literature, for hookworm (Traub et al. 2008), *Ascaris* (Traub et al. 2002), *Giardia* (Hopkins et al. 1997; Read et al. 2002; Sulaiman et al. 2003) and *Blastocystis* (Clark 1997), respectively.

7.3.5 Statistical analysis

The data were double-entered and validated in EpiData (www.epidata.dk). Statistical analysis was carried out using STATA version 12 (StataCorp LP; College Station, TX). We calculated the cumulative prevalence for all IPIs. Potential risk factors were determined by matching the parasite infection status of humans and animals with corresponding questionnaire data, either at individual or household level. Possible risk factors were determined by regression analysis and were considered significant at p-levels smaller than 0.05.

7.4 Results

7.4.1 Study population

In this study, 67 households were enrolled, yielding 218 participants, of which 99 (45.4%) were male and 119 (54.6%) were female, with an average age of 30 years (range 2–84 years). Almost half (n = 107, 49.1%) of all participants worked as farmers in rice fields, while many others (n = 89, 40.8%) were pre-school or school-aged children. Altogether, 94 dogs were sampled; 39 (41.5%) were male and 55 (58.5%) were female, with an average age of 27 months. The dogs were further classified into puppies/ juveniles (<12 months old), adults (1–6 years old) and geriatrics (> 6 years old) (Inpankaew et al. 2007). Seventy-six pigs were sampled; 35 (46.1%) were male and 41 (53.9%) were female, with an average age of 6.9 months. The pigs were classified into 10 piglets (<1 month), 41 juveniles (>1-8 months) and 25 adults (>8 months) (Yui et al. 2014).

7.4.2 IPI prevalence in humans

The major IPIs found in humans were hookworms (63.3%), *Entamoeba* spp. (27.1%), *S. stercoralis* (24.3%), *G. duodenalis* (22.0%) and *Blastocystis* (18.4%) (Table 1). In total, 14 different parasites were diagnosed, including eight helminthic and six protozoan parasites. Of the 218 participants, 27 (12.8%) were negative in all examinations. More than a quarter of the human participants (64, 29.4%) were infected with at least one parasite and a third (72, 33.0%) with two or more parasites. Three (1.4%) and one (0.5%) participant(s) harboured five and six parasites, respectively (Figure 7.1). In Figure 7.2, the prevalences of parasites (those with the highest infection rates) are given for the different age-groups. For hookworm, the prevalence increases from less than 50.0% in children up to the age of ten to more than 60.0% in adolescents and then remains above 60.0% in all subsequent age-groups. For *S. stercoralis*, the prevalence also increases over age, reaching its peak in age-groups 30 years and older. Figure 7.3 shows that the average number of helminthic co-infections

increases over age, whereas the average number of protozoan co-infections is highest in children and lowest in adults older than 51 years.
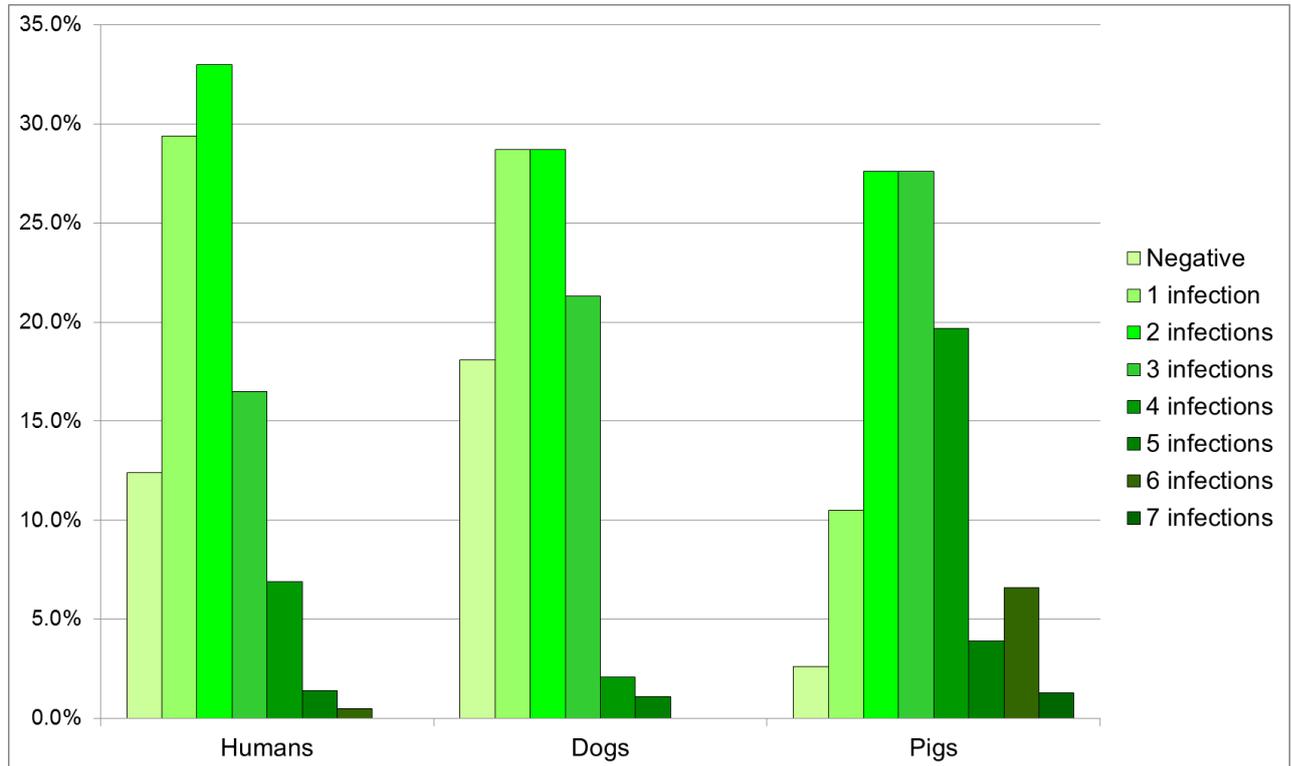
Figure 7.1 – Prevalence of multi-parasitism in humans, dogs and pigs
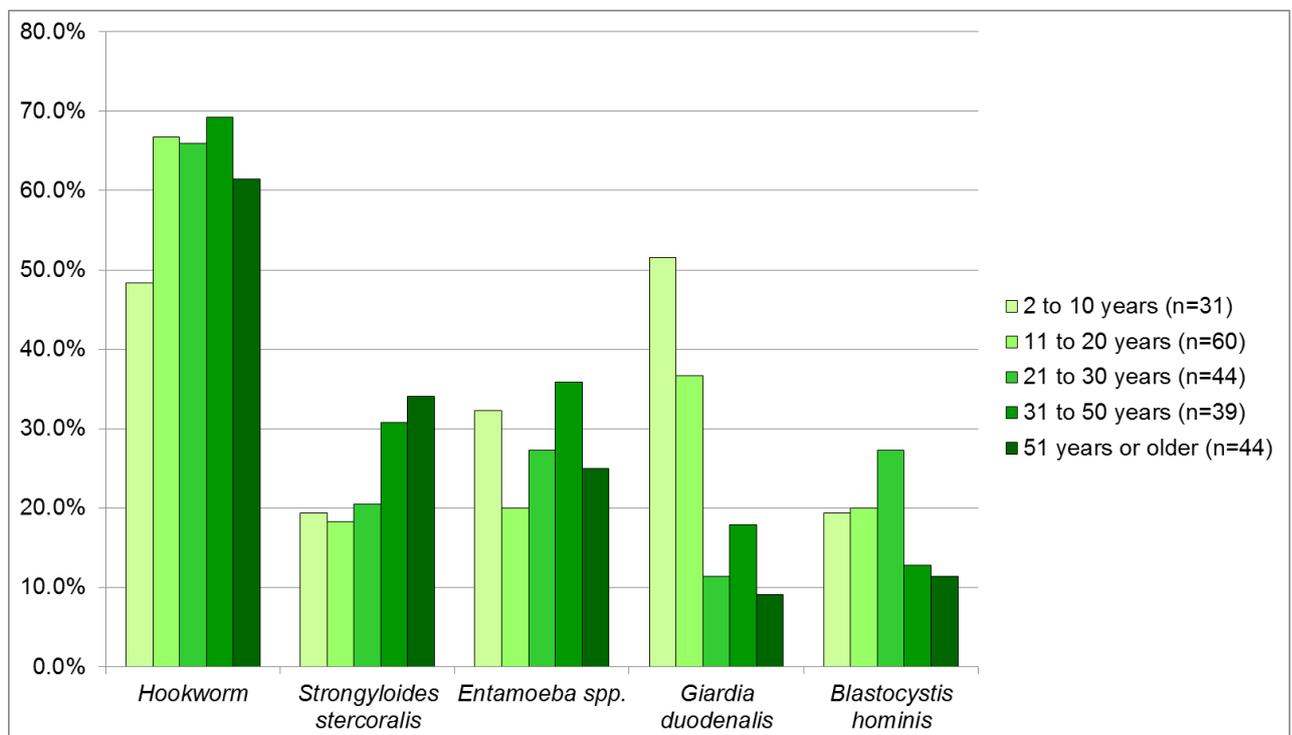
Figure 7.2 – Cumulative prevalence of intestinal parasitic infection in humans by age-group
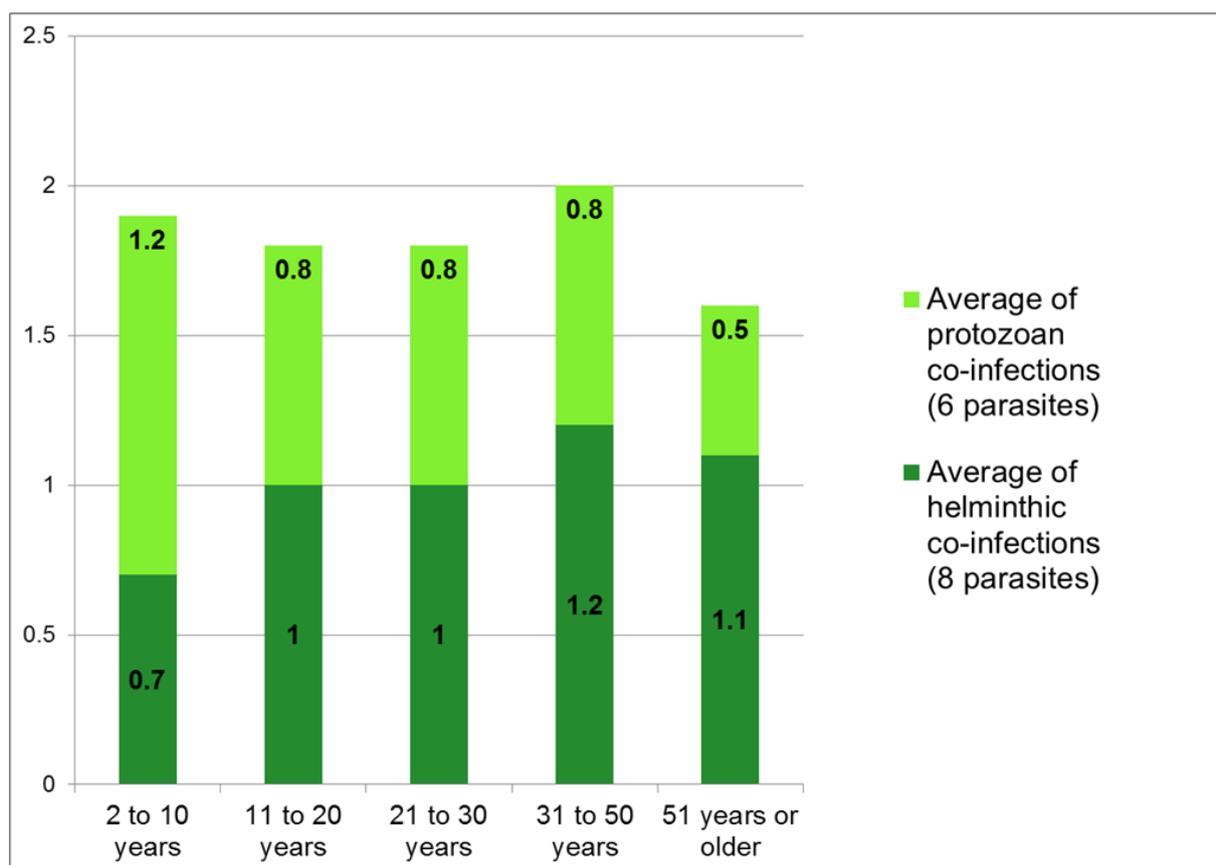
Figure 7.3 – Number of human protozoan & helminth co-infections in humans by age-group

7.4.3 Prevalence in animals

The most frequent IPIs found in dogs were hookworm (80.8%), followed by *Spirometra* spp. (21.3%) and *Strongyloides* spp. (14.9%, Table 2). Seventy-seven (81.9%) dogs had at least one or more IPIs. One dog (1.1%) was co-infected with five parasites. Multiple parasitic infections were observed in more than half (53.2%) of the dogs (Fig. 1). Eleven species of parasites (eight helminths and three protozoa) were identified in dogs, seven of which have zoonotic potential (hookworms, *Strongyloides* spp*., Trichuris* spp., *Toxocara canis*, *Echinostoma* spp., *G. duodenalis* and *Entamoeba* spp.).

The most common parasites found in pigs were *Isospora suis* (75.0%), *Oesophagostomum* spp. (73.7%) and *Entamoeba* spp. (31.6%, Table 2). Of the 76 pigs, 74 (97.4%) had at least one parasitic infection and 66 (86.8%) had multiple infections (Figure 1). A single pig (1.3%) had co-infections with seven IPIs. Altogether, twelve different parasitic infections were identified (eight helminthic and four protozoan infections) five of which are known for their zoonotic potential (*Ascaris* spp*., Trichuris* spp*., Capillaria* spp., *Balantidium coli* and *Entamoeba* spp.).

Table 7.1 – Cumulative prevalence of intestinal parasitic infections in humans

Parasite	Kato Katz	Average EPG (range)	Flotation	PCR	Baermann	Koga Agar	FECT	Total infected (n=218)
Nematodes								
Hookworm	46 (21.1)	611 (24-12576)	58 (26.6)	124 (56.9)	0	41 (18.8)	18 (8.3)	138 (63.3)
Strongyloides stercoralis	0	0	4 (1.8)	n.a.	43 (19.8)	35 (16.1)	3 (1.4)	53 (24.3)
Ascaris spp.	0	0	0	5 (2.3)	0	0	0	5 (2.3)
Trichuris trichiura	0	0	2 (0.9)	n.a.	0	0	0	2 (0.9)
Cestodes								
Taenia spp.	2 (0.9)	1368 (816-1920)	1 (0.5)	n.a.	1 (0.5)	0	1 (0.5)	4 (1.8)
Trematodes								
Small Trematode Eggs	2 (0.9)	36 (24-48)	0	n.a.	0	0	9 (4.1)	11 (5.1)
Large Trematode Eggs	0	0	0	n.a.	0	0	3 (1.4)	3 (1.4)
Dicrocoelium dentriticum	0	0	0	n.a.	0	0	1 (0.5)	1 (0.5)
Protozoa								
Entamoeba spp.	0	0	34 (15.6)	n.a.	0	0	41 (18.8)	59 (27.1)
Giardia duodenalis	0	0	20 (9.2)	40 (18.4)	0	0	21 (9.6)	48 (22.0)
Blastocystis hominis	0	0	0	n.a.	0	0	40 (18.4)	40 (18.4)
Endolimax nana	0	0	0	n.a.	0	0	15 (6.9)	15 (6.9)
Isospora spp.	0	0	10 (4.6)	n.a.	0	0	0	10 (4.6)
Iodamoeba bütschlii	0	0	7 (3.2)	n.a.	0	0	8 (3.7)	8 (3.7)

Table 7.2 – Cumulative prevalence of intestinal parasitic infections in dogs and pigs

Parasite	Dogs (n=94)	Pigs (n=76)
Nematodes		
Ascaris spp.	n.a.	20 (26.3)
Ascarops spp.	n.a.	2 (2.6)
Capillaria spp.	n.a.	4 (5.3)
Eucoleus spp.	3 (3.2)	n.a.
Gnathostoma doloresi	n.a.	7 (9.2)
Hookworm	76 (80.9)	n.a.
Metastrongylus spp.	n.a.	15 (19.7)
Oesophagostomum spp.	n.a.	56 (73.7)
Spirocerca lupi	3 (3.2)	n.a.
Strongyloides spp.	14 (14.9)	n.a.
Strongyloides ransomi	n.a.	8 (10.5)
Toxocara canis	6 (6.4)	n.a.
Trichuris suis	n.a.	15 (19.7)
Trichuris vulpis	9 (9.6)	n.a.
Cestodes		
Spirometra spp.	20 (21.3)	0
Trematodes		
Echinostoma spp.	2 (2.1)	0
Protozoa		
Balantidium coli	0	12 (15.8)
Eimeria spp.	0	5 (6.6)
Entamoeba spp.	6 (6.4)	24 (31.6)
Giardia duodenalis	2 (2.1)	0
Isospora canis	13 (13.8)	n.a.
Isospora suis	n.a.	57 (75.0)

7.4.4 Risk factors

The results of the personal questionnaire were used to determine possible risk factors for IPI. Questions related to personal hygiene practices showed that 195 (89.5%) and 198 (90.8%) participants reported washing their hands after defecating and before eating, respectively. Almost half (104, 47.7%) reported using soap to wash their hands. Almost all participants (215, 98.6%) reported possessing shoes, yet only 79 (36.7%) said that they wore them all the time. More than half of the participants (120, 55.1%) reported using latrines.

Comparing age and infection status, we found that individuals older than 30 years were twice as likely to be infected with *S. stercoralis* (OR: 2.02, 95% CI: 1.03 – 3.97) than other age groups. Children and adolescents up to the age of 20 were almost four times more likely to be infected with *G. duodenalis* (OR: 3.76, 95% CI: 1.82 – 7.94). The regression analysis for helminths showed that individuals that did not use shoes while defecating had an increased risk of hookworm infection (p=0.038; OR: 6.0, 95% CI: 1.1 – 28.6). Infection with *Taenia* lead to an increased risk for epigastralgia (p=0.009; OR: 3.83, 95% CI 1.4 – 10.6).

Questionnaire data on the animals were used to gain insight into how the animals were kept. All dog-owners reported that their dogs (94, 100%) were allowed to roam freely and to defecate indiscriminately throughout the community. The majority (77.7%) of owners never removed dog faeces from their property and only 11 (11.7%) removed the faeces on a daily basis. Of the owners that did collect dog faeces, 21 (22.3%) disposed of the excrement in an area behind the house. Only two (2.1%) dogs were seen by a veterinarian on at least one occasion, but none had been dewormed.

Almost all (67, 88.2%) pigs were kept in the backyard of the house and also defecated there. Only 23 (30.3%) pig owners reported removing the faeces daily, while 27 (35.5%) owners had never removed the faeces. Of those owners who removed faeces, two-thirds (32, 65.3%) said they disposed of the faeces outside the pig stable, yet not one owner reported composting the faeces. Most owners reported using pig faeces as fertilizer, either for their fish ponds (38, 50.0%), their vegetable gardens (22, 29.0%) or their rice fields and fish ponds combined (9, 11.8%). Nine (11.8%) of the pigs had been attended to by a veterinarian, and six (7.9%) had been previously dewormed.

7.5 Discussion

The present study showed similar patterns of IPIs in humans compared to previous surveys conducted in Cambodia (Khieu et al. 2013a; Khieu et al. 2013b; Koga-Kita 2004). However, in this study, no cases of *Ascaris* spp. in humans were detected by microscopy. This coincides with the findings of Park and colleagues (Park et al. 2004). We demonstrated that in all age-groups, the average number of co-infections is about the same, yet helminthic co-infections accumulated over time, with a peak in 30–50 year old individuals. The trend for protozoan co-infections is reversed, with the highest number of protozoan co-infections occurring in children. This pattern might reflect higher exposure of children. Alternatively, it could be because of higher infection intensities rather than prevalence in children, as microscopy can miss low-intensity protozoan infections (Traub et al. 2009), although this applies also for helminthic infections.

Our study results show that IPIs are common in both dogs and pigs in rural Cambodian villages. Several of these IPIs have zoonotic potential, including *G. duodenalis* (Traub et al. 2004a), *Entamoeba* spp. (Dado et al. 2012), hookworm (Areekul 1979), *T. canis* (Colli et al. 2010), *Strongyloides* spp. (Viney and Lok 2007), and *Echinostoma* spp. (Lan-Anh et al. 2009) in dogs; and *Entamoeba* spp. (Verweij et al. 2001), *Ascaris* spp. (Anderson 1995), *Trichuris* spp. (Nissen et al. 2012), *B. coli* (Owen 2005) and *Capillaria* sp. (Fuehrer et al. 2011) in pigs. It is likely that similar patterns of parasitism in these animals can be found elsewhere in the country. The village sampled in our study is not far from the border with Lao PDR. Yet, while fish-borne zoonotic trematodes are abundant in Lao PDR (Forrer et al. 2012; Sayasone et al. 2011; Sayasone et al. 2009), this was not the case in our study, as the habit of eating raw fish is virtually non-existent in Khmer communities. No comparable studies have appeared for Cambodia, however the high prevalence of canine and pig IPIs, coupled with poor sanitation and hygiene practices and a lack of veterinary attention, are conducive to the transmission of zoonotic parasites. Even if it is not possible to extrapolate the results from one single village to the whole province and/or country, they show the need for integrated national education programmes on controlling parasites, not only in humans but also in domestic animals.

Dogs are associated with more than 60 zoonotic parasites worldwide, many of which pose serious public health concerns (Eguia-Aguilar et al. 2005). Compared with some other studies in South Asian countries, the overall prevalence of IPIs in dogs in Cambodia was higher (81.9%) than previously reported for dogs in rural India, for example (Traub et al. 2002). Our interviews revealed that dogs in rural Cambodian villages such as Dong village are largely kept as guard dogs and allowed to roam freely, especially during the day. The dogs are also allowed inside the house and around rice and vegetable fields and ponds. At night-time, the dogs then often stay in or around the house. Dogs, therefore, pose a serious zoonotic risk as they have the potential to transmit zoonotic parasites through their close association with household members as well as through heavy contamination of the environment, including soil, fresh produce and waterways, with parasite eggs and oocysts.

In contrast, pigs in Cambodia are typically bred and reared for home consumption, although they are frequently sold at the local market. Most pigs are raised in stables, but contamination of the environment with their manure is substantial, as it is used by the villagers to fertilize their crops and fish ponds. The pig faeces used have not been composted. It is uncertain if such practices represent a risk for transmitting fish-borne zoonotic parasites, for which pigs are known hosts (Lan-Anh et al. 2009). Further, it is not clear to what extent pig manure handling and storage practices inactivate any parasites present.

It was not possible to assess statistically significant risk factors for parasitic infection in animals, as most animals shared common behavioural features and management practices. This, coupled with very high infection rates made it impossible to compare risks between groups and instead we offer a more descriptive picture of how dogs and pigs are kept in rural Cambodian villages.

While pointing out the zoonotic potential of parasites, it must be acknowledged that, for several parasites, human-to-human transmission is still the main transmission route. Furthermore, for some parasites, clinical consequences in non-immunocompromised populations are not well established, i.e. for *Blastocystis hominis*.

In conclusion, this is the first study to describe the prevalence of IPIs among humans, dogs and pigs living in the same community in rural Cambodia. We offer a descriptive assessment of the infection status of humans and animals in one village. Further large-scale studies would be needed to extrapolate the results to the national level. Several parasite species in both humans and animals could not be identified by the morphological characteristics of eggs or cysts alone (e.g. hookworms and *Entamoeba*). Furthermore, infected animals were not available for necropsy. Future studies to identify potentially zoonotic IPIs in animals and humans within these communities, using molecular methods, will further shed light on the role that domestic animals play in transmitting IPIs.

7.6 Acknowledgements

7.7 Author Contributions
FS, TI, RJT, AD and PO conceived and designed the study; FS, TI, and VK collected data under the supervision of SM; TI analysed the animal stool specimens; HM coordinated the

field laboratory activities; WC, CC, DS, SS and SM coordinated the field work in Cambodia; FS, TI, RJT, AD and PO analysed data and interpreted the results; FS and TI wrote the manuscript with RJT and PO; PO supervised the first author in all aspects of the study; all authors have read and approved the final version of manuscript.

7.8 Conflict of interest

The authors declare that no competing interest exists

7.9 Financial Support

8. High prevalence *of Ancylostoma ceylanicum* hookworm infections in humans, Cambodia, 2012

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8.1 Abstract

Ancylostoma ceylanicum, a hookworm of canids and felids in Asia, is emerging as the second most common hookworm of humans. A 2012 epidemiological survey in rural Cambodia revealed 57.4% of the population was infected with hookworms; of those, 51.6% with *A. ceylanicum* hookworms. As with *Necator americanus* hookworm infections in this community, the greatest intensities of *A. ceylanicum* eggs were in persons 41-50 years of age. Over 90% of dogs within this community also harboured *A. ceylanicum* hookworms. Characterization of the cytochrome oxidase-1 gene divided isolates of *A. ceylanicum* hookworms into 2 groups, 1 containing isolates from humans only and the other a mix of isolates of human and animal. We hypothesize that preventative chemotherapy in the absence of concurrent hygiene and animal health programs may be a factor leading to emergence of *A. ceylanicum*; thus, we advocate for a One Health approach toward the control of this zoonosis.

Keywords: hookworm, *Ancylostoma ceylanicum*, zoonosis, Cambodia, humans

8.2 Introduction

Human hookworm infections with *Necator americanus* and *Ancylostoma duodenale* hookworms continue to be recognised as a leading cause of iron deficiency anemia and protein malnutrition in developing countries (Crompton 2000). On the basis of parasitological surveys of faecal samples, hookworms are estimated to infect 576–740 million people globally, and over half the infections occurring in Asia and the Pacific regions (Bethony et al. 2006). Recent molecular-based epidemiological surveys have shown *Ancylostoma ceylanicum* to be the second most common hookworm species infecting humans in Asia. In Thailand Laos, and Malaysia, 6% - 23% of individuals positive for hookworm eggs were found infected with *A. ceylanicum* helminths (Conlan et al. 2012; Jiraanankul et al. 2011; Ngui et al. 2012; Traub et al. 2008). There are an estimated 19-73 million *A. ceylanicum* hookworm-infected persons in regions where this zoonotic helminth is known to be endemic (Traub 2013). Dogs and cats act as natural reservoirs for hookworm transmission to humans, and the prevalence of *A. ceylanicum* hookworms in these animals ranges from 24% to 92% in the Asia-Pacific region (Ngui et al. 2012; Scholz et al. 2003; Traub et al. 2007; Traub et al. 2008). Much like anthroponotic hookworms, *A. ceylanicum* hookworms have the potential to produce clinical symptoms of ground itch (a pruritic popular hypersensitivity response caused by the entry of helminthes into the skin), epigastric pain, diarrhoea and anaemia in humans (Carroll and Grove 1986; Chung et al. 2012; Hsu and Lin 2012; Wijers and Smit 1966; Yoshida 1971). However, despite these reports, relatively little is known about the clinical significance and infection dynamics of this zoonotic hookworm in humans, dogs and cats. Differentiation of the genus of hookworms in humans is imperative because each species vary in its biology, life cycle, pathophysiology, and epidemiology, and these differences have key implications when assessing hookworm-associated illnesses and establishing control measures.

The internal transcribed spacer (ITS) -1 and -2 regions and the 5.8S region have been used to detect and characterize hookworm infections directly from eggs in human and animal faeces (Ngui et al. 2012; Sato et al. 2010; Traub et al. 2008; Traub et al. 2004b). In addition, sequencing of the cytochrome c oxidase subunit 1 (cox-1) gene has been successfully used to establish intraspecies genetic differences of many strongylid nematodes, including hookworms (Hawdon et al. 2001; Hu et al. 2002; Ngui et al. 2013; Zhan et al. 2001).

The aim of our study was to determine the prevalence, associated risk factors, and infection dynamics of hookworm species infection in humans and dogs living in a rural Cambodian village. To carry out this investigation, we used a combination of conventional parasitologic and molecular epidemiologic approaches.

8.3 Materials and methods

8.3.1 Study site and sample collection

The study was conducted in May 2012 in Dong, a rural village in Rovieng District, Preah Vihear Province, Cambodia. Preah Vihear Province is located in northern Cambodia, bordering Thailand and Laos (13°47'N 104°58'E). The climate is tropical; temperatures are warm and hot all year round, and seasons alternate between dry and wet. Subsistence farming (rice, vegetables, and fish) constitutes the primary source of income for the community. Drinking water is sourced from wells, well pumps, and rain water tanks, and just over half the households own a latrine. All household electricity is battery or generator powered. Approximately half the households feed semi-domesticated, free-roaming community dogs. These dogs are allowed to defecate indiscriminately within the village or outside the homes of their owners. All study participants responded to a questionnaire covering demographics, dietary habits, personal hygiene, and level of household income and assets.

The study protocol was approved by the Ethics Committee of the Canton of Basel and Baselland (EKBB), Switzerland, and the National Ethics Committee Health Research, Ministry of Health, Cambodia. Dong, the village selected for study, had previously been categorized as having endemic soil-transmitted helminths (Khieu et al. 2013b). According to the treatment guidelines of the Cambodian helminths control program, all children attending primary school in the village were administered albendazole (400 mg) and mebendazole (500 mg) twice a year. At completion of the study, all participants who were found positive for *Strongyloides* spp. were treated with ivermectin (200 µg/kg body weight), and participants infected with other soil-transmitted helminths were treated with albendazole (400 mg).

A cross-section of 67 households was randomly selected from a list provided by the Dong village authority. A total of 218 persons from those households were enrolled in the study. Of the 218 persons, 99 (45.4%) were male. The average age of participants was 30.0 years (range 2–84); female participants were marginally older, on average, than male participants (30.3 vs. 29.8 years of age). On the first day of the study, informed consent was obtained from the enrolled participants and questionnaires were administered by interviewing. Interviews with children (i.e., participants 2–17 years of age) were conducted with the assistance of a parent or legal guardian.

Pre-labeled stool containers were distributed to the 218 study participants for collection of feces on the second morning of the study. Fecal samples were collected from participants'

dogs (N = 94), when applicable. Samples (≈3–5 g) from dogs were collected directly from the rectum at time of the participant interview and placed into a sterile plastic container. If insufficient stool was obtained from a dog, the animal was confined within the owner's property and re-sampled on the second morning of the study. All fecal samples were chilled immediately in a cool box and transported to a laboratory in Rovieng Health Center (Rovieng District, Preah Vihear Province, Cambodia) within 2 h after collection. After fecal samples arrived at the laboratory, a minimum of 2 g of each sample was placed into a 15-mL centrifuge tube containing 10% formaldehyde for parasitologic analysis, and 1–2 g of each sample was placed into a 15-mL centrifuge tube containing 2.5% potassium dichromate for molecular analysis. These samples were then shipped at room temperature to the School of Veterinary Science, University of Queensland, Gatton, Queensland, Australia, for further analysis.

8.3.2 Parasitological procedures

All fecal samples were examined by microscope. The relative intensity of hookworm infection, in eggs per gram, was determined by flotation, using a sodium nitrate solution (specific gravity 1.20) (Inpankaew et al. 2007).

8.3.3 DNA extraction

Genomic DNA was extracted directly from fecal samples by using the PowerSoil DNA Isolation Kit (Mo Bio, Carlsbad, CA, USA) according to the manufacturer's instructions, with the exception that fecal samples were subjected to a 5-min disruption by using 0.5-mm Zirconia/Silica beads (BioSpec Products, Inc., Bartlesville, OK, USA) instead of the beads provided by the manufacturer. Final elution of DNA was made in 100 µL of elution buffer. The extracted DNA was stored at -20°C until required for PCR amplification.

8.3.4 PCR and DNA sequencing of hookworms from humans

PCR was conducted by using primers RTHW1F and RTHW1R (Palmer et al. 2007; Traub et al. 2004b) in 25-µL volumes; each final reaction contained 1× CoralLoad PCR Buffer (QIAGEN Pty Ltd, Hilden, Germany), 12.5 pmol of each primer, 0.5 U of HotStar Taq DNA Polymerase (QIAGEN), and 2 µL of DNA. The cycling conditions were the same as the published protocol (Palmer et al. 2007; Traub et al. 2004b) except for an initial denaturation of 5 min at 95°C. A positive control of *N. americanus* and *A. ceylanicum* hookworms and negative controls of distilled water were included in each run. PCR amplicons that were ≈380 bp in size, corresponding to *Ancylostoma* spp. hookworms, were purified by using the

PureLink Quick PCR Purification Kit (Life Technologies Corporation, Carlsbad, CA, USA) and submitted to the University of Queensland Animal Genetics Laboratory, Gatton, for bidirectional DNA sequencing.

8.3.5 Characterization of hookworms in dogs

PCR–restriction fragment length polymorphism (RFLP) characterization of hookworms from dogs was carried out as described (Palmer et al. 2007; Traub et al. 2004b). In brief, RTGHFI and RTABCR1 primers were used to amplify a 545-bp region of ITS-1, 5.8S, and ITS-2 of *A. caninum*, *A. ceylanicum*, and *Uncinaria stenocephala* hookworms. In a separate PCR, a 673-bp region of *A. braziliense* hookworm was amplified by using RTGHF1 and a specific reverse primer, RTAYR1. Both PCR reactions consisted of 1× CoralLoad PCR Buffer (QIAGEN), 12.5 pmol of each primer, 0.2 µL of 20 mg/mL Bovine Serum Albumin, 2 µL DNA, and 1 U of HotStar Taq Polymerase (QIAGEN) in a 25-µL reaction. The cycling conditions were as published (Palmer et al. 2007; Traub et al. 2004b), except for an initial denaturation time of 5 min at 95°C. Amplified PCR product (10 µL; RTGHF1/RTABCR1) was digested with HinFI and RsaI endonucleases in separate reactions at 37°C for 3 h. The RFLP patterns generated by each sample were then compared to the expected RFLP profiles for each hookworm species.

8.3.6 PCR and DNA sequencing of cox-1 for *A. ceylanicum* hookworm

Samples from dogs and humans that were positive for *A. ceylanicum* hookworms were further characterized to a haplotype level by analysis of the mitochondrial gene (cox-1). AceyCOX1F (5'-GCTTTTGGTATTGTA-AGACAG-3') and AceyCOX1R (5'-CTAACAACATAATAAG-TATCATG-3') were specifically designed to amplify a 377-bp region of the cox-1 gene of *A. ceylanicum* hookworm. The PCR was carried out in 25-µL volumes, with each reaction containing 1× CoralLoad PCR Buffer, 12.5 pmol of each primer, 0.5 U of HotStar Taq DNA polymerase, and 2 µL of DNA. The cycling conditions were 95°C for 5 min, followed by 50 cycles at 94°C for 30 s, 58°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 7 min. A positive control of *A. ceylanicum* hookworm and a negative control of distilled water were included in the run. PCR-positive samples were purified by using the PureLink Quick PCR Purification Kit according to the manufacturer's protocol. Bidirectional DNA sequencing was performed by the University of Queensland Animal Genetics Laboratory.

8.3.7 Phylogenetic analyses

DNA sequences were analyzed by using the Finch TV version 1.4.0 trace viewer (Geospiza, Inc., Seattle, WA, USA) and aligned by using BioEdit version 7.2.0 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) together with the cox-1 gene sequences from the following hookworm species: *A. ceylanicum* Malaysia isolates (GenBank accession nos. KC247727– KC247745, Pos Iskandar [Human] and Sg Bumbun [Human]); *A. caninum* and *A. duodenale* (GenBank accession nos. NC012309 and NC003415, respectively); and *A. ceylanicum* Thailand genotype (GenBank accession no. KF896595). Neighbor-joining analyses were conducted by using Tamura-Nei parameter distance estimates, and the tree was constructed by using Mega4.1 (www.megasoftware.net). Bootstrap analyses were conducted using 1,000 replicates.

8.3.8 Statistical analyses

We used STATA version 12 (StataCorp LP, College Station, TX, USA) for data entry and statistical analyses. The prevalence of hookworm infection was calculated by using descriptive statistics for microscopy and molecular results. A univariate model was used to assess potential risk factors associated with hookworm infection, including the Odds Ratios (ORs) and 95% confidence interval (CI) were reported. The level of statistical significance was set at $p<0.05$. Factors that were significant in univariate analysis were evaluated by multivariate analysis, when applicable.

8.4 Results

8.4.1 Prevalence of hookworm infections

The prevalence of hookworm infection among the 218 persons tested in Dong village was 26.6% (58/218) as determined by microscopic examination and 57.4% (124/218) as determined by PCR based on amplification of the partial ITS gene. Among dogs, 80.8% (76/94) were positive for hookworms by microscopic examination, and 95.7% (90/94) were positive by PCR based on amplification of the partial ITS gene.

8.4.2 Molecular characterization of hookworm species

Of the 124 persons with positive samples, 64 (51.6%) harboured *A. ceylanicum* hookworms; 57 (89.0%) of these infections were single infections. An equal percentage of persons, 64

(51.6%), were infected with *N. americanus* hookworms, mostly as single infections (59/64 [92.2%]), and 4 (3.2%) persons were infected with *A. duodenale* hookworms (Table 8.1).

Of the 90 dogs with positive samples, 85 (94.4%) were infected with *A. ceylanicum* hookworms, mostly (81/85 [95.3%]) as single infections, and 8 (8.9%) were infected with *A. caninum* hookworms. One dog was found shedding *N. americanus* eggs (Table 8.1).

Table 8.1 – Hookworm species found in humans and dogs

Infected host, hookworm species	No. (%) positive
Human	
N. americanus	59 (47.6)
A. ceylanicum	57 (46.0)
A. duodenale	1 (0.8)
N. americanus & *A. ceylanicum*	4 (3.2)
A. ceylanicum & *A. duodenale*	2 (1.6)
N. americanus & *A. ceylanicum* & *A. duodenale*	1 (0.8)
N. americanus & *A. duodenale*	0
Total	124 (100)
Dog	
A. ceylanicum	81 (90.0)
A. caninum	5 (5.6)
A. ceylanicum & *A. caninum*	3 (3.3)
A.ceylanicum & *N. americanus*	1 (1.1)
Total	90 (100)

8.4.3 Phylogenetic analysis of cox-1 gene of *A. ceylanicum*

Of 68 human and 82 dog samples positive for hookworms, 28 (41.2%) and 65 (79.3%), respectively, were successfully amplified at the cox-1 gene. Of these, 21 human and 27 dog PCR-positive amplicons were randomly selected for DNA sequencing and subsequent phylogenetic analysis.
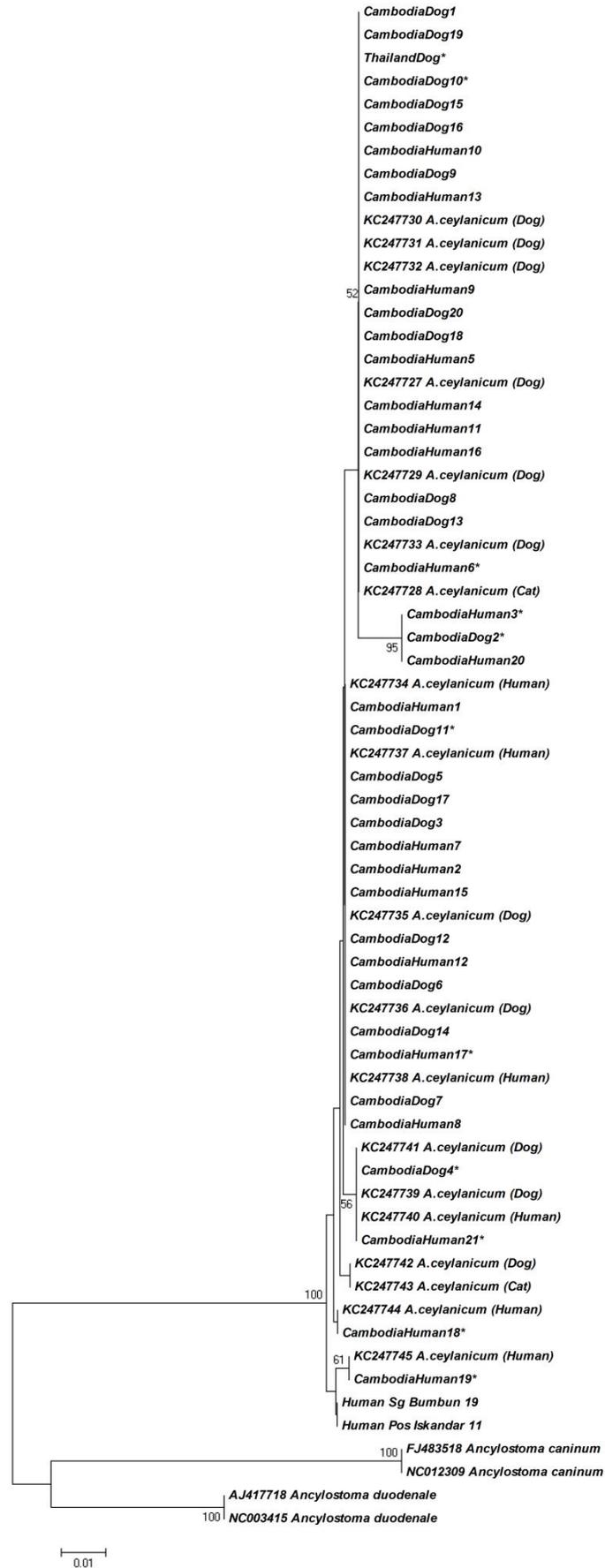
Figure 8.1 – Phylogenetic tree of *A. ceylanicum* from 21 humans and 27 dogs

The phylogenetic tree distinctly separated into 3 clusters; the *A. ceylanicum* hookworm isolates grouped together and were genetically distinct from *A. caninum* hookworm isolates (GenBank accession nos. NC012309 and FJ483518) and *A. duodenale* hookworm isolates (GenBank accession nos. NC003415 and AJ417718). Within *A. ceylanicum* hookworm isolates, there was strong bootstrap support (100%) for the division of isolates from various geographic locations into 2 clades. The first clade comprised 4 human isolates, 1 from the current study in Cambodia (Human 19) and 3 previously reported human isolates from Malaysia (GenBank accession no. KC772445; [Human [Sg Bumbun]; Human [Gurney] and Human [Pos Iskandar]). The second clade comprised a mix of isolates from humans (n = 20) and dogs (n = 27) from villages in Cambodia; humans (n = 5), dogs (n = 11), and cats (n = 2) from Malaysia (Ngui et al. 2013); and 1 dog in Thailand (GenBank accession no. KF896595). For human- and dog-derived *A. ceylanicum* hookworms, representative DNA sequences at each cox-1 haplotype were submitted to GenBank under accession nos. KF896596 – KF896605 (Figure 8.1 - see sequences marked with asterisks)

8.4.4 Age-related prevalence and intensity of *N. americanus* and *A. ceylanicum* hookworm infections

The prevalence of *N. americanus* hookworms peaked in persons 31–50 years of age, whereas the prevalence of *A. ceylanicum* hookworms peaked in persons 15–20 years age and again in persons 31–50 years of age (Figure 1). The highest egg intensities for single infections attributed to *N. americanus* and *A. ceylanicum* hookworms occurred in persons 21–30 years of age (Figure 8.2).

8.4.5 Risk factors associated with hookworm infection of humans and dogs

The results of regression analysis showed an increased risk for hookworm infection in persons who did not wear shoes while defecating (odds ratio 6.0, 95% CI 1.1–28.6; p = 0.038). No significant associations were found between the prevalence and intensity of hookworms by age group, sex, household income, or dietary practices.
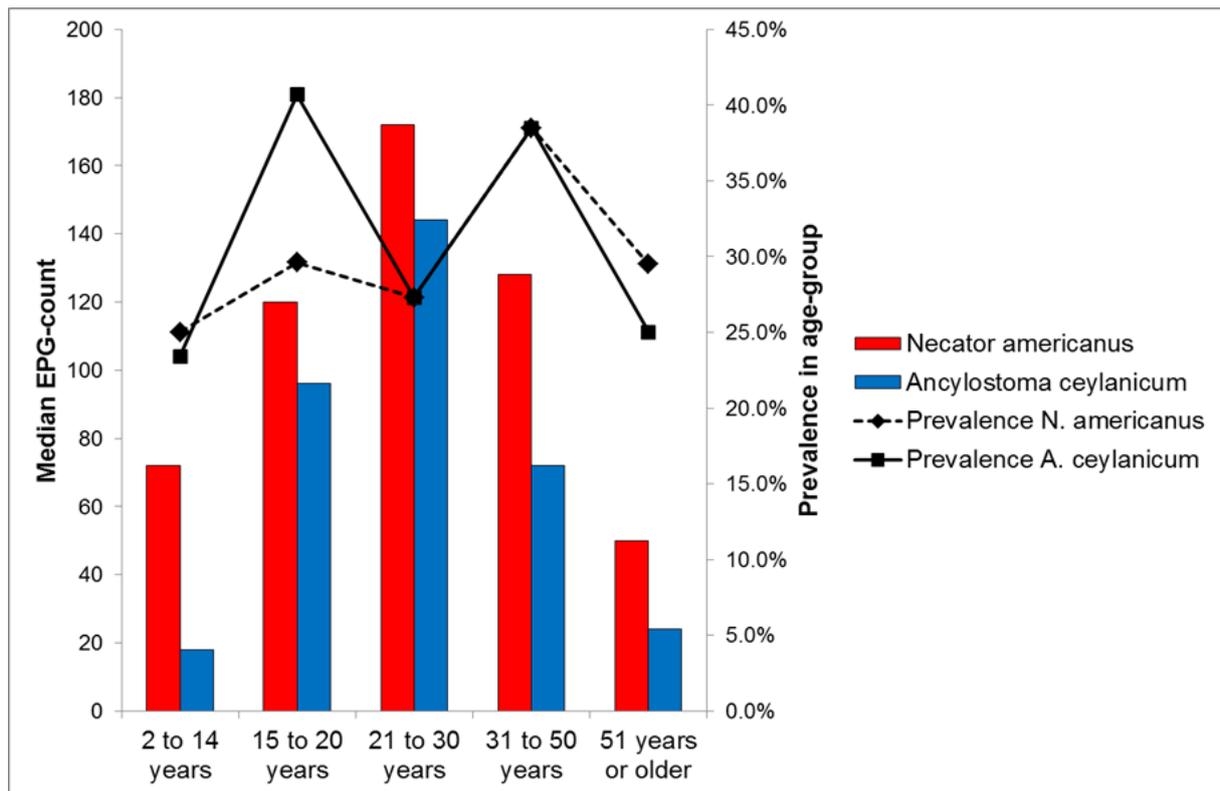
Figure 8.2 – Prevalence and intensity of *N. americanus* and *A. ceylanicum* in humans, stratified by age-group

8.5 Discussion

In this study, zoonotic ancylostomiasis caused by *A. ceylanicum* hookworms was found to be highly endemic among humans in Dong village, Preah Vihear Province, Cambodia; community dogs were the likely zoonotic reservoir. This finding is in stark contrast to other molecular-based prevalence studies in the region that have consistently demonstrated *N. americanus* to be the predominant hookworm species in humans, followed by *A. ceylanicum* and *A. duodenale* hookworms (Conlan et al. 2012; Jiraanankul et al. 2011; Ngui et al. 2012; Traub et al. 2008). PCR proved a superior alternative to microscopy-based techniques for the detection of hookworms in fecal samples (Gasser et al. 2008; van Mens et al. 2013). In Dong village, the prevalence of *A. ceylanicum* hookworms matched that of its anthroponotic counterpart, *N. americanus* hookworms, and infections with *A. ceylanicum* hookworms substantially out-numbered those with *A. duodenale* hookworms. In addition, most infected persons harbored single-species hookworm infections; just over 10% of hookworm-positive persons had mixed-species infections. These results raise questions about the potential infection dynamics between hookworm species within individual hosts. Our study supports an earlier hypothesis (Traub 2013) that anthroponotic hookworms may

have a cross-protective role in expelling and preventing the subsequent establishment of *A. ceylanicum* hookworms via a Th2 response (Croese and Speare 2006). The major immunologic acting against incoming L3 and L4 hookworm infection is regulated by the infection itself (Behnke et al. 1997). Thus, the presence of a stable and long-lived (3–6 years) infection with anthroponotic species may play a role in providing an unsuitable environment for the establishment of incoming larvae of another closely related (albeit potentially shorter-lived and suboptimally host adapted) species — in this case, *A. ceylanicum* hookworms. Reduced burdens of anthroponotic hookworm species may also have the added advantage of easing density dependent intraspecific competition for limited resources within the intestinal niche (Paterson and Viney 2002), leading to an opportunistic establishment of *A. ceylanicum* hookworms. Although data on the natural life span of *A. ceylanicum* hookworms in humans does not exist, infections in Dutch servicemen 5 months after their return from New Guinea (Anten and Zuidema 1964a) suggest that chronic infections with this hookworm may occur.

The initiating or causal factor for the emergence of highly endemic levels of monospecific infections with *A. ceylanicum* hookworms in Dong village remains unclear. In this study, potential causal factors for human infection are likely related to the high levels of *A. ceylanicum* hookworm infections in community dogs. In rural Malaysia, close contact with community dogs and cats was shown often to be associated with human infection with *A. ceylanicum* hookworms (Ngui et al. 2012). In Dong village, dogs were reported to defecate indiscriminately in environments shared with humans, leading to widespread environmental contamination with infective *A. ceylanicum* hookworm larvae. For humans, defecating while bare foot was shown to be the most significant risk factor for infection with both species of hookworms. Whether these factors, coupled with the administration of preventative chemotherapy, led to an increased opportunity for the *A. ceylanicum* hookworm to replace the niche of its anthroponotic competitors remains unanswered. Either way, integrated control programs aimed at combining chemotherapeutic interventions with improvements in community hygiene and animal health programs will aid in curbing this potentially opportunistic zoonosis.

Molecular epidemiologic data gathered from characterization of the cox-1 gene of *A. ceylanicum* hookworms strongly support previous findings (Ngui et al. 2013) that *A. ceylanicum* hookworm isolates from humans and animals formed 2 genetically distinct groups, 1 comprising isolates specific to humans and the other comprising isolates from humans, dogs, and cats. Most *A. ceylanicum* hookworm isolates from humans in Dong village clustered within the zoonotic haplotype, confirming that transmission from dogs to humans has occurred. Genetic groups inferred by the cox-1 gene of *A. ceylanicum*

hookworms were found to be independent of geographic source. Whether the 2 primary haplotypes differ in biologic, epidemiologic, and pathophysiologic characteristic warrants further investigation.

The transmission dynamics of *A. ceylanicum* hookworms in humans of different ages largely paralleled that of *N. americanus* hookworms: persons 21–30 years of age excreted the highest number of eggs. This highly unexpected result leads to important inferences. First, the result suggests that the previous classification of *A. ceylanicum* as an abnormal and minor hookworm of humans (Chowdhury and Schad 1972) no longer stands. Second, monospecific infections of humans with <100 *A. ceylanicum* worms have been reported to cause anemia, even in well-nourished persons (Anten and Zuidema 1964b; Chung et al. 2012). Attention therefore must be directed this zoonosis as a major cause of human illness in areas where this zoonosis is endemic.

The zoonotic helminth *A. ceylanicum* can no longer be classified as an abnormal hookworm of humans. Although previous studies have reported its emergence as the second most common human hookworm species in Southeast Asia, our study demonstrate its ability to infect humans at prevalence and intensity levels at par with that of its anthroponotic competitor, the *N. americanus* hookworm. We hypothesize that expansion of preventative chemotherapy in the absence of concurrent hygiene and animal health programs to be a potential causal factor for the emergence of this zoonosis. Attention, therefore, must be directed to the human health impact of this zoonosis, and a One Health approach should be adopted for its control.

8.6 Acknowledgements

9. Evaluation of real-time PCR for *Strongyloides stercoralis* and hookworm as diagnostic tool in asymptomatic schoolchildren in Cambodia

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9.1 Abstract

Diagnosis of soil-transmitted helminths such as *Strongyloides stercoralis* and hookworms (*Ancylostoma duodenale* and *Necator americanus*) is challenging due to irregular larval and egg output in infected individuals and insensitive conventional diagnostic procedures. Sensitive novel real-time PCR assays have been developed. Our study aimed to evaluate the real-time PCR assays as a diagnostic tool for detection of *Strongyloides* spp. and hookworms in a random stool sample of 218 asymptomatic schoolchildren in Cambodia.

Overall prevalence of 17.4% (38/218) and 34.9% (76/218) were determined by real-time PCR for *S. stercoralis* and hookworms, respectively. Sensitivity and specificity of *S. stercoralis* specific real-time PCR as compared to the combination of Baermann/Koga Agar as gold standard were 88.9% and 92.7%, respectively. For hookworm specific real-time PCR a sensitivity of 78.9% and specificity of 78.9% were calculated. Co-infections were detectable by PCR in 12.8% (28/218) of individuals.

Strongyloides stercoralis real-time PCR applied in asymptomatic cases showed a lower sensitivity compared to studies undertaken with symptomatic patients with the same molecular tool, yet it proved to be a valid supplement in the diagnosis of STH infection in Cambodia.

Keywords: Real-time PCR, *Strongyloides stercoralis*, hookworm, sensitivity

9.2 Introduction

Soil-transmitted helminths (STH) are the cause of the most neglected diseases worldwide (Hotez et al. 2006). STH are prevalent in tropical and sub-tropical regions with high prevalence rates in areas with poor hygiene and sanitation. In particular *S. stercoralis* and the hookworms *Necator americanus* and *Ancylostoma duodenale* represent clinically relevant members of STH. *S. stercoralis*, an intestinal nematode, infects an estimated 30-100 million people worldwide (Bethony et al. 2006). In most cases it causes asymptomatic infections. However, severe and life-threatening infections known as hyper-infection syndrome may occur (Marcos et al. 2008). Chronic infection with the hookworm may cause anemia and protein deficiency in both adults and children (Stoltzfus et al. 1997).

Traditionally, diagnosis is accomplished by microscopic examination of stool samples and culture methods. However, in light infected individuals microscopy remains insensitive and culture is time consuming (Agrawal et al. 2009). Sensitivity considerably increases when more than one sample is analyzed (Knopp et al. 2008; Marti and Koella 1993). Use of consecutive samples on up to four days is therefore recommended (Dreyer et al. 1996). Importantly, parasite density in the majority of *S. stercoralis* cases is low, and therefore it would be desirable to increase the sensitivity of diagnostic methods. Diagnosis of hookworm infection is not as challenging due to higher parasite concentrations. Complicating, multiple infections with different STH can occur rendering simultaneous detection of different helminths a useful expansion of diagnostic tests. Novel molecular methods have been described (Kramme et al. 2011; Verweij et al. 2007; Verweij et al. 2009). Verweij et al recently presented data of a pilot study conducted in Ghana describing a novel *S. stercoralis* real-time polymerase chain reaction (PCR) and a novel hookworm specific real-time PCR (Verweij et al. 2007; Verweij et al. 2009). These assays displayed a high analytical sensitivity and proved their principle. We validated a described *Strongyloides* spp. and hookworm real-time PCR assay in asymptomatic Cambodian children using a gold standard of microscopy and culture techniques.

9.3 Materials and Methods

9.3.1 Ethical consideration

The study was integrated into a larger study on *S. stercoralis* infection and risk factors (Khieu et al. 2013a) approved by the ethics committee of the cantons of Basel-Stadt and Basel-Land (EKBB, number 21/09, dated 29 January 2009), Switzerland, and the National Ethics

Committee for Health Research (NECHR, number 033, dated 20 March 2009), Ministry of Health, Cambodia. Children with a positive diagnostic result for *S. stercoralis* and hookworm by microscopy were treated with ivermectin (200 µg/kg BW divided in two doses, over two days) and mebendazole (500 mg, single dose), respectively.

9.3.2 Sample collection

During a field study in the semi-rural province of Kandal, south of Phnom Penh in Cambodia in 2009, 501 stool samples were collected from children from four different schools. The stool samples were transported to the laboratory within 2 h after collection and subsequently analyzed by Baermann (Baermann 1917), Koga Agar (Koga et al. 1991) and Kato-Katz (Katz et al. 1972) standard techniques. Kato Katz slides were read starting 30 minutes after preparation, to allow clearing time for the hookworm eggs. Koga Agar slides were read after incubation at 28°C for 48 hours. In addition, an aliquot of stool was taken and immediately frozen at -20°C and shipped to Basel, Switzerland, for further analysis. A total of 218 samples were randomly selected (114; 52.3% male versus 104; 47.3% female, average age 10.6 years) and analyzed by real-time PCR assays.

9.3.3 DNA extraction from stool samples

For isolation of nucleic acids from stool samples the QIAmp DNA stool kit (Qiagen, Hilden, Germany) was used according to the manufacturer's instructions. 200 mg of stool was used as sample input volume, elution volume was 200 µl.

9.3.4 Real-time PCR assays

Real-time-PCR assays for *S. stercoralis*, *A. duodenale* and *N. americanus* were done according to Verweij et al. with minor modifications (Verweij et al. 2007; Verweij et al. 2009). In brief, a 25 µl monoplex-reaction contained 5 µl DNA template, 12,5 µl HotStarMastermix (Qiagen), 3mM $MgCl_2$, 400 nM of each sense and antisense primer (TIB-Molbiol, Berlin, Germany) and 200 nM of the respective probe (TIB-Molbiol). All probes were labelled with the dye FAM and BBQ as quencher. Thermal cycling on an ABI 7500 Realtime PCR System (Applied Biosystems, Weiterstadt, Germany) comprised denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. Fluorescence was measured during the 60°C step.

9.3.5 Construction of DNA standards

A 101 base pair (bp) real-time amplicon of the 18S rRNA gene of *S. stercoralis,* a 70 bp fragment of the ITS-2 gene of *A. duodenale* and a 100 bp fragment of the ITS-2 gene of *N. americanus* was ligated into pCR2.1 plasmid vector and cloned into *Escherichia coli* using the pCR 2.1 TOPO TA cloning kit (Invitrogen, Karlsruhe, Germany) as described (Panning et al. 2008). Resulting plasmids of the correct length were purified by means of a QIAprep Spin Miniprep kit (Qiagen) and DNA concentration was spectrophotometrically measured. Each of the plasmids was used as a run control at a concentration of 100 DNA copies/PCR reaction. No-template controls were included in each run.

9.3.6 PCR inhibition control

Inhibition was monitored by spiking the *S. stercoralis* plasmid at a concentration of 100 copies/real-time PCR reaction in each duplicate sample.

9.3.7 Cross reactivity panel

DNA of the following parasites was tested with each real-time PCR to assess a possible cross reaction: *A. duodenale, N. americanus*, *Trichuris trichiura*, *Ascaris suum*, *Trichinella spiralis, Toxocara canis, Wuchereria bancrofti, Loa loa, Dirofilaria immitis, Echinococcus granulosus*, *Schistosoma mansoni,* and *S. stercoralis.*

9.3.8 Data analysis

Results of parasitological and real-time PCR examinations were double-entered into EpiData version 3.1 (EpiData Association, Odense Denmark) and Microsoft Excel, respectively. After validation, analysis was performed with Stata version 10 (Stata Corporation, Texas USA).

S. stercoralis real-time PCR were compared with results of Baermann and Koga Agar methods (gold standard) of the same stool sample. Results of Kato-Katz technique were regarded as gold standard for the hookworm real-time PCR assays. Standard statistical techniques were applied where appropriate. A *p*-value of less than 5% was regarded as statistically significant.

9.4 Results

All real-time PCR assays were optimized with respect to sensitivity and specificity. Ten DNA plasmid copies per reaction of *A. duodenale, N. americanus*, and *S. stercoralis*, respectively, were detectable on a regular basis by each individual real-time PCR. No unspecific amplification with other related parasites (*Trichuris trichiura*, *Ascaris suum*, *Trichinella spiralis, Toxocara canis, Wuchereria bancrofti, Loa loa, Dirofilaria immitis, Echinococcus granulosus*, *Schistosoma mansoni*) was detectable (data not shown).

9.4.1 Validity of *S. stercoralis* PCR

Overall, real-time PCR detected *Strongyloides* DNA in 38 of 218 (17.4%) samples whereas Baermann and Koga Agar combined detected *S. stercoralis* larvae in 41 of 218 (18.8%) samples (p=0.851). 25 of 38 (65.8%) real-time PCR positive samples were also positive by Baermann and/or Koga Agar methods (Table 9.1). Real-time PCR failed to demonstrate DNA in two Baermann/Koga Agar positive and in one Baermann positive patient. In 17 Koga Agar positive samples only 4 (23.5%) were also positive by real-time PCR. Stool samples of 164 study participants were negative in all three methods. None of the sample contained substances inhibitory to real-time PCR as demonstrated by amplification of the spiked external inhibition control. Sensitivity and specificity of real-time PCR as compared to the combination of Baermann/Koga Agar methods as gold standard were 88.9% and 92.7%, respectively (Table 9.1).

Table 9.1 – Comparison of results obtained by Baermann and Koga Agar and real-time PCR for the detection of *S. stercoralis*

N	Microscopy		PCR		Sensitivity	Specificity
	Baermann	Koga Agar	Positive	Negative	%	%
18	+	+	16	2	88.9	
6	+	-	5	1	83.3	
17	-	+	4	13	23.5	
41	+/-	+/-	25	16	61.0	
177	-	-	13	164		92.7

Ct-values obtained for the real-time PCR positive samples were compared between Baermann/Koga Agar positive samples, Baermann positive/Koga Agar negative samples, Baermann negative/Koga Agar positive and Baermann/Koga Agar negative samples, respectively. Ct-values were low in samples with parasitological positive results and high in samples with negative results: Mean and median Ct-values were 31.9, 38.2, 35.0 and 39.1 and 33.0, 39.0, 34.5 and 39.0, respectively. A statistically significant difference (p<0.05) was observed between Baermann/Koga Agar positive and Baermann positive only samples and for Baermann/Koga Agar positive and Baermann/Koga Agar negative samples (p=0.012; Figure 9.1).
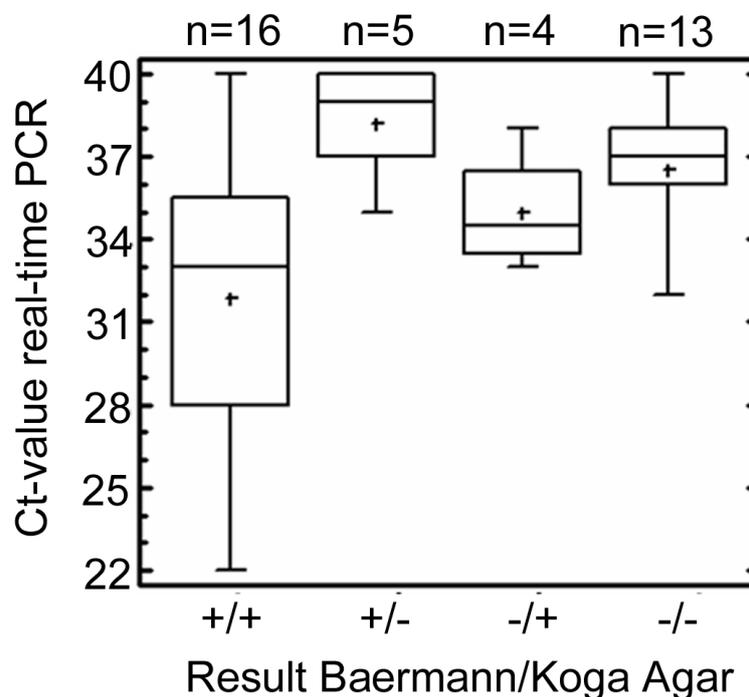
Figure 9.1 – Comparison of Ct-values of *S. stercoralis* real-time PCR results with Baermann and/or Koga Agar positive and negative samples, respectively. Y-axis denotes Ct-values of real-time PCR

9.4.2 Validity of hookworm PCR

A total of 76 of 218 (34.9%) samples yielded a positive result by real-time PCR of which 52 (23.9%) samples were positive by the Kato-Katz method (p=0.016). 41 of 52 (78.8%) of the Kato-Katz positive samples were positive by real-time PCR. Interestingly, 35 of 218 (16.1%) samples yielded a positive real-time PCR result without detection of eggs. Of note, 16 of these 35 (45.7%) samples yielded a Kato-Katz positive result when two further stool samples of these children were examined. Finally, 131 of 218 (60.1%) samples

demonstrated to be negative by all methods used. The overall analysis yielded a sensitivity of 78.9% and specificity of 78.9% of real-time PCR (Table 8.2).

Table 9.2 – Comparison of results obtained by Kato-Katz and real-time PCR methods for the detection of hookworms

N	Microscopy	PCR		Sensitivity	Specificity
	Kato Katz	Positive	Negative	%	%
52	+	41	11	78.9	
166	-	35	131		78.9

Ct-values of *N. americanus* real-time PCR positive samples were lower in Kato-Katz positive (median Ct-value 36.5) than Kato-Katz negative samples (median Ct-value 37.0), however no statistical difference was recorded (p=0.17). Ct-values of *A. duodenale* real-time PCR positive samples were not done due to its low prevalence.

Real-time PCR was used for the identification of hookworm species. A total of 56 of 76 (73.7%) hookworm real-time PCR positive samples were positive for *N. americanus* specific real-time PCR only. In particular five of 76 (6.6%) were positive for *A. duodenale* specific real-time PCR only and 15 of 76 (19.7%) samples yielded positive results for both *N. americanus* and *A. duodenale* specific real-time PCR.

Among the 56 *N. americanus*-DNA positive samples half (28, 50.0%) were concomitantly positive by microscopy. Only 1 of 5 *A. duodenale*-DNA positive sample (20.0%) was also positive by microscopy.

Twelve of 15 (80.0%) samples with positive results for *N. americanus* and *A. duodenale*-specific real-time PCR were also positive by Kato-Katz (Table 2)

9.4.3 Samples with multiple detections

Strongyloides spp. and hookworm co-infections were detected in 26 of 218 (11.9%) samples by classical parasitological methods. Twenty-three of these positive samples (88.5%) yielded a real-time PCR positive result. In detail, 13 (50.0%) yielded positive results by both real-time PCR for *S. stercoralis* and hookworms; 2 (7.7%) were positive by *S. stercoralis* real-time

PCR only; 8 (30.8%) samples were positive by hookworm real-time PCR only; 3 (11.5%) were negative by both real-time PCR.

Interestingly, 11 of 15 (73.3%) Koga Agar positive samples for *S. stercoralis*, which tested negative by *Strongyloides* real-time PCR yielded a hookworm positive result in the Kato-Katz method. In addition, 4 of 11 (36.4%) hookworm positive samples *Strongyloides* larvae were found in Koga Agar method.

9.5 Discussion

STH have a significant impact on public health in affected countries (Bethony et al., 2006) (Olsen et al. 2009). Many parts of Southeast Asia provide the ideal ecological and economical background for high infections rate of *S. stercoralis* and hookworms. Reports from Cambodia and Laos PDR showed prevalence rates as high as 20.2% for *S. stercoralis* (Chhakda et al. 2006) and 76.8% for hookworms (Sayasone et al. 2011). Therefore, precise and reliable diagnostic methods remain crucial for public health interventions. Recently, molecular methods have been established for *Strongyloides* spp. and hookworms (Verweij et al. 2007; Verweij et al. 2009). Using the *S. stercoralis* positive results of Baermann and/or Koga Agar, our validation of real-time detection of DNA for *Strongyloides* spp. and hookworms revealed a sensitivity and specificity of 61.0% and 92.7% and 78.9% and 78.9%, respectively. These values for sensitivity of *S. stercoralis* detection are similar to the values obtained by Verweij et al (61.1% and 92.4%) (Verweij et al. 2007; Verweij et al. 2009).

When comparing the coprological diagnostic methods with the real-time PCR, different factors have to be considered. First, the sample input volume of culture methods and of real-time PCR assays differs by a factor of 10, which considerably increases sensitivity for the culture method. Increasing input volume for real-time PCR is not an option since stool contains substances which may be inhibitory to real-time PCR. However, real-time PCR inhibition was not an issue in our samples as no significant inhibition of the external amplification control was observed. As reported earlier and confirmed in our study use of consecutive samples increased sensitivity by a factor of 1.5 for *Strongyloides* as well as for the hookworms by conventional methods (Khieu et al. 2013a; Marti and Koella 1993). It is likely that repeated testing of consecutive sample by real-time PCR will also increase the detection rate. However, this is not an option in resource limited settings but might be considered in in-depth epidemiological studies.

Second, in our study only asymptomatic patients were included. It is reasonable to assume that these patients do not excrete *S. stercoralis* larvae and hookworm eggs in high concentrations. Symptomatic infections are more likely to be of higher parasite burden, thus increasing the number of detectable cases. This is also reflected by the overall high Ct-values in our study indicating rather low parasite burden. Of note, data on the correlation between Ct-values and intensity of infection has not been established and further studies are needed.

Technically, for maximum sensitivity we used a monoplex real-time PCR approach in contrast to Verweji et al. This further minimizes the risk of false-positive results as multiplex real-time PCR assays are prone to complex oligonucleotide/probe interactions. Analytical sensitivity, as demonstrated on dilution series of plasmid DNA, was comparable to other assays used for parasite detection. However, we can not rule out that infections with low parasite numbers were missed by this approach due to clumped occurrence (Poisson distribution) of the parasites. Interestingly, Wichmann and colleagues have recently demonstrated that detection of free parasite DNA in serum is possible for *Schistosoma* spp. and might constitute a feasible alternative for pathogen detection (Wichmann et al. 2009). Given the life cycles of *Strongyloides* and hookworm infections this might also be a feasible approach and deserves further studies.

Although real-time PCR in particular seems to be a valid technique for diagnosis its application in resource poor settings remains limited due to rather expensive laboratory equipment. Koga Agar culture and/or Baermann technique can be performed at the village level in the field. They have yielded superior performance in this study. However, given the importance of multiple parasitic infections in endemic countries molecular techniques become more advantageous. Especially multiplex real-time PCR methods as proposed by Verweij and colleagues seem to be a valid and cost effective way in diagnosing multiple infections (Verweij et al. 2007). Of note, experience with microscopy and conventional methods is waning in many laboratories in industrialized countries. In this respect, for *S. stercoralis* sensitivity of real-time PCR in comparison to the Koga Agar method was disappointing. One reason might by a possible misidentification of larvae. Further studies are needed if molecular methods can compensate for this.

To conclude, we could demonstrate that real-time PCR assays can be applied in asymptomatic cases but sensitivity was slightly lower compared to culture and/or microscopy. Further studies are needed to determine the applicability of real-time PCR in the clinical as well as in a research setting.

10. *Strongyloides stercoralis* **larvae excretion pattern before and after treatment**

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10.1 Summary

The variability of larvae excretion impedes the parasitological diagnosis of *Strongyloides stercoralis* in infected individuals. We assessed the number of larvae excreted per gram (LPG) stool in 219 samples from 38 infected individuals over seven consecutive days before and in 470 samples from 44 persons for 21 consecutive days after ivermectin treatment (200 µg/kg BW). The diagnostic sensitivity for a single stool sample was about 75% in individuals with low-intensity infections (<1 LPG) and increased with higher infection intensity to 95% (>10 LPG). Doubling the number of samples examined per person increased sensitivity to more than 95%, even in low-intensity infections. Comparison of local maxima in excreted larvae with random numbers showed no indication for a cyclic excretion of larvae. After treatment all individuals stopped excreting larvae within 3 days. In none of the following 18 days (total 388 Baermann and 388 Koga Agar tests) larvae were detected. Two stool samples collected on consecutive days are recommended in settings where low or heterogeneous infection intensities are likely. Taking into account a possible biological variability in excretion, the efficacy of ivermectin treatment can therefore be assessed already four days after treatment.

Key Words: *Strongyloides stercoralis*, Larvae excretion, Sensitivity, Treatment, Ivermectin

Key Findings:

A higher infection intensity increases the sensitivity for diagnosis of *S. stercoralis*

Sensitivity of diagnosis for *S. stercoralis* is increased by analysing multiple stool samples

Treatment with ivermectin is highly effective

Three days post-treatment with ivermectin, no more *S. stercoralis* larvae are detectable in faecal samples

10.2 Introduction

Strongyloides stercoralis, a soil-transmitted nematode of world-wide distribution (Bethony et al. 2006; Genta 1989), is arguably the most neglected of all neglected helminth infections (Olsen et al. 2009; Schär et al. 2013b). Infectious *S. stercoralis* (L_3) larvae are found in humid soil contaminated with faecal material. From there, the larvae penetrate the intact skin of the human host. In resource-poor countries, environmental, socio-economic and behavioral factors, and inadequate sanitary conditions provide ideal circumstances for high transmission rates of *S. stercoralis.*

Parasitological diagnosis of *S. stercoralis* infection relies on identification of larvae in stool. The Baermann technique (Baermann 1917) and Koga Agar culture (Koga et al. 1991) are recommended, preferably combined and carried out on multiple stool samples (Khieu et al. 2013a). Infections with low numbers of excreted larvae are particularly challenging and can easily be missed. For other intestinal helminths, detailed egg excretion pattern studies were performed, e.g. for liver flukes (Lovis et al. 2012), but information is missing for *S. stercoralis.*

The efficacy of ivermectin for the treatment of *S. stercoralis* has been demonstrated many times (Adenusi 2003; Heukelbach et al. 2004a; Igual-Adell et al. 2004; Khieu et al. 2013a; Marti et al. 1996; Ordonez and Angulo 2004; Suputtamongkol et al. 2011; Toma et al. 2000). However, little is known about the parasite clearance time and stop of larvae excretion after ivermectin treatment (Uparanukraw et al. 1999) in endemic settings. Yet, this information is crucial for planning and execution of follow-up examinations after treatment.

We assessed the excretion pattern of *S. stercoralis* larvae in a daily follow-up seven days before and 21 days after ivermectin treatment (200 µg/kg BW, single oral dose) in 38 infected patients.

10.3 Materials and methods

10.3.1 Ethical considerations

This study was undertaken in Preah Vihear (March 2010) and Takeo province (March 2011) in Cambodia. The study protocol was approved by the Ethics Committee of the Canton of Basel (EKBB, 21/09 dated 29 January 2009) Switzerland and the National Ethics Committee Health Research (NECHR; 033 dated 20 March 2009), Ministry of Health, Cambodia. All persons were informed on the purpose and process of the study. Written informed consent

was obtained from all individuals prior to enrollment. All diagnosed infections were treated at the end of the study according to the National Cambodian treatment guidelines.

10.3.2 Enrollment

Individuals diagnosed with *S. stercoralis* during a community based helminth survey were randomly selected. Either the examined person alone (in Preah Vihear) or all persons living in the same household (in Takeo) were enrolled.

10.3.3 Field procedures

Each morning for seven days, the participants provided a stool sample (Figure 1) and at the same time received a new labeled stool container for the following day. The collected samples were transported at ambient temperature within an hour to the field laboratory in a health center where the laboratory examinations were conducted (2-3 hours after defecation).

Participants positive for *S. stercoralis* at least once during the seven days were treated with a single oral dose of ivermectin 200µg/kg body weight on day seven. The daily stool collection was then continued for another 21 days after treatment.

10.3.4 Laboratory procedures

Upon arrival of the sample in the laboratory a quantitative Baermann test was performed. (Baermann 1917). In brief, the stool containers with the sample were weighted. A Baermann test was set up and the containers were weighted again afterwards. The stool sample (about 15-25 grams) was placed in a wire-mesh in a funnel filled with tap water. Artificial light was used to stimulate larval migration out of the stool into the funnel. After two hours the water containing the larvae was collected and centrifuged for 5 minutes (2000 rpm). The entire sediment was examined under a microscope (400x magnification) and *S. stercoralis* larvae were counted.

During follow-up after treatment, for each stool sample a Koga Agar culture was performed in addition (Koga et al. 1991). In brief, a hazelnut-sized piece of stool was placed on a soft Agar plate. They were then incubated for 48 hours at 28°C. Then the plates were rinsed and the liquid centrifuged for 2 minutes at 2000 rpm and the entire sediment examined for presence of larvae and adults.

10.3.5 Statistical analysis

Data was entered in Microsoft Excel, cross-checked against original data sheets and analyzed using the statistical software environment R. *S. stercoralis* infection intensity was calculated as larvae per gram stool (LPG) per person and day. The relationship between diagnostic sensitivity and infection intensity (mean LPG per individual) were visually assessed using a lowess smother. Diagnostic sensitivity was calculated for examining a stool samples on a single day, for examining samples collected on two consecutive days and for examining samples collected one two alternating days, respectively. For participants with six and more stool examinations before treatment the larvae excretion pattern was examined in detail. We compared the larvae excretion counts maxima within individuals with the pattern observed in randomly generated numbers to identify potential cyclic patterns. The LPGs per day are compared, and a local maximum is defined when the LPG of one day is higher than the LPG sum of the day before and after combined.

For the follow-up after treatment the infection rate was calculated for each day post-treatment as the number of patients in which at least one *S. stercoralis* larva was observed divided by the number of patients treated and followed-up. For each observation day the larvae reduction rate was calculated. We used the mean number of larvae excreted by each individual before treatment as the reference value. The overall larvae reduction rate was calculated by first dividing the mean LPG for each day post treatment by the overall LPG. The result of the division was subtracted from one to get the larvae reduction rate.

10.4 Results

10.4.1 Enrollment and compliance

Altogether 65 persons were enrolled in the study (Figure 10.1). The overall mean age was 26.7 years (range: 3-81 years). There were 32 (49.2%) male and 33 (50.8%) female participants. We examined a total of 800 stool samples with an average weight of 19.7 grams (range: 4.3 – 44.7 grams) with the Baermann method.

10.4.2 Larval excretion pattern before treatment

A total of 39 (60.0%) persons were infected with *S. stercoralis.* During baseline examination 18 (27.7%) provided seven samples, 14 (21.5%) provided six samples and 32 (49.2%) provided two to five samples, respectively. One person (2.6%) provided only one sample and

was excluded from the excretion pattern analysis. The mean number of stools provided by healthy individuals was significantly lower than by infected persons (4.2 *versus* 5.6 stool samples, p = 0.008). Altogether 26 persons were negative, yet one person who was negative on the initial examination presented a *S. stercoralis* infection on the third day. It subsequently was moved into the treatment group.

For the larval excretion pattern analysis, we used the 38 infected persons which provided more than 1 sample (median: 6 samples). A considerable variation in larvae excretion densities was observed in the pretreatment examinations. The highest and lowest average density per person observed were 151.2 LPG (range: 28.8 – 410.6 LPG) and 0.03 LPG (range 0 – 0.07 LPG), respectively.
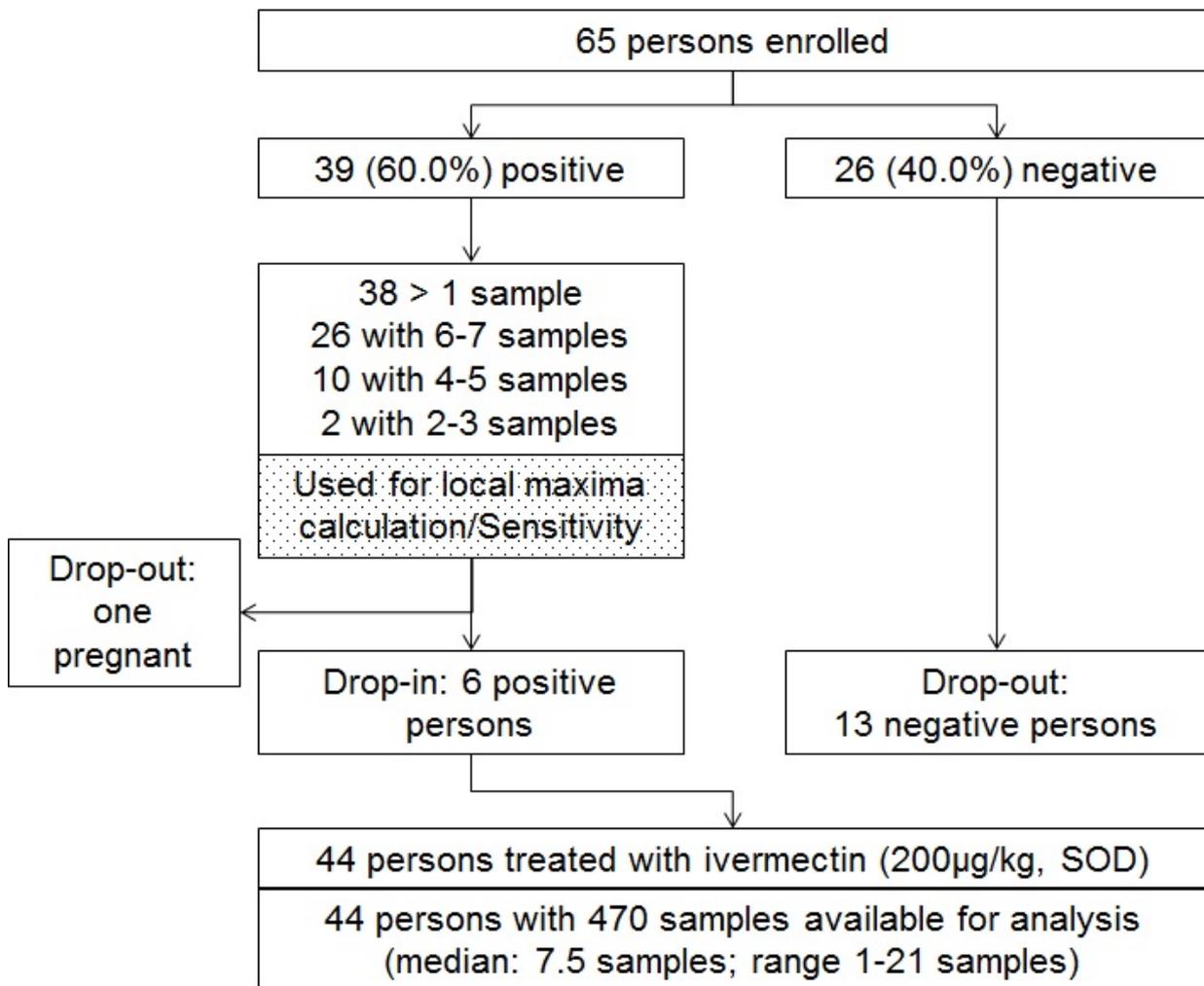
Figure 10.1 – Flowchart: enrolment and follow-up of study participants

Twenty-three patients (60.5%) had a positive result on all days before treatment, 9 (23.7%) had one day where no larvae were excreted, four (10.5%) two days with no larvae present and two (5.3%) had three negative examinations.

The diagnostic sensitivity for a single day examination was 75% for individuals having an average LPG of 1 or less. A higher average LPG increased the sensitivity. An LPG of 10 or more resulted in sensitivities of above 95% (Figure 2). When stool samples collected on two days were analyzed, the sensitivity remarkably increased. The sensitivity for two samples collected on consecutive days was 97.0% (LPG <= 1) and close to 100% (LPG > 10), respectively. When two samples with one sampling free day in-between were analyzed, the sensitivity was close to 100%, for low-intensity infections and high-intensity infections alike.
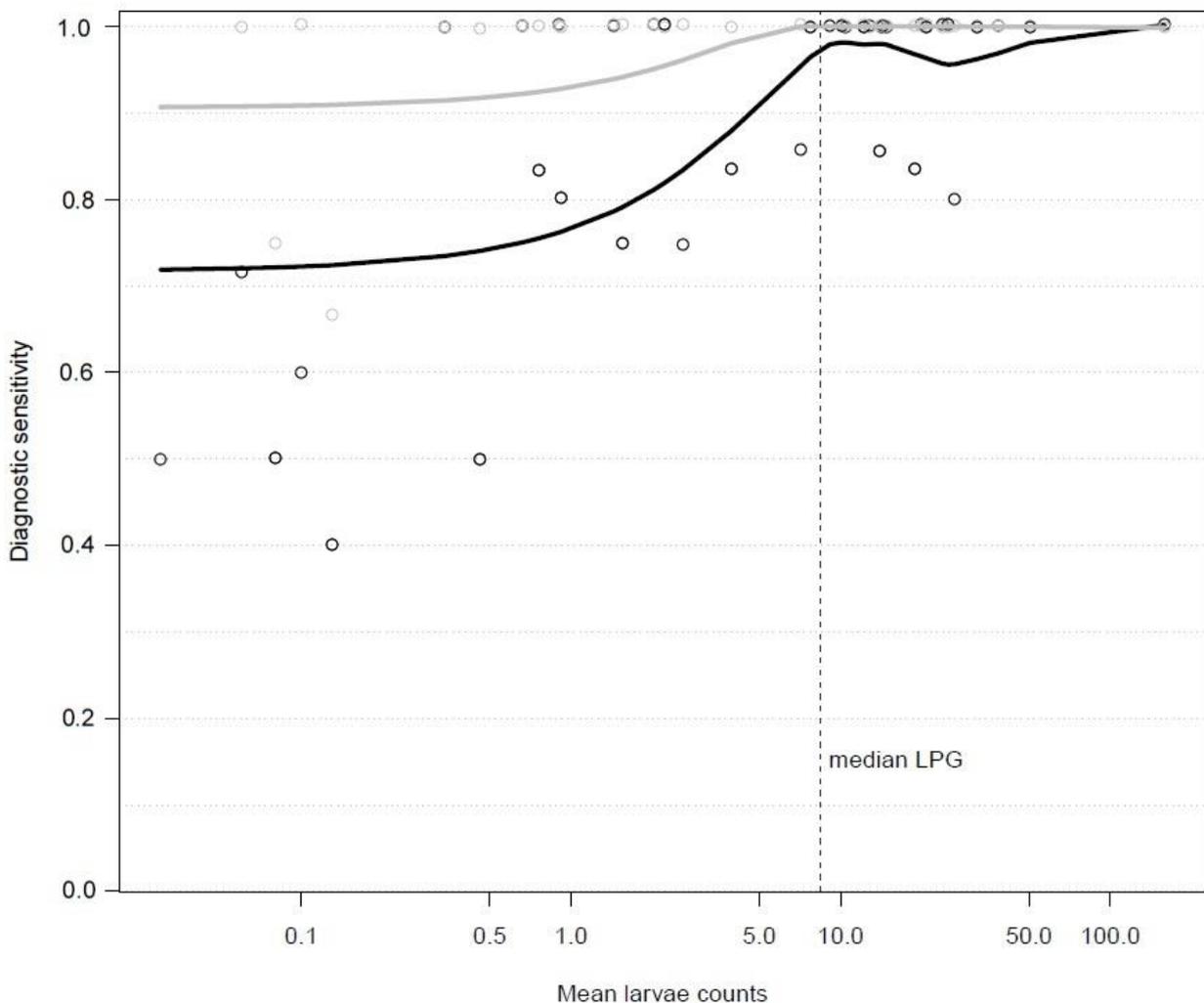
Figure 10.2 – Diagnostic sensitivity of examining a single stool sample (black dots) and two samples collected on consecutive days (grey dots) by mean LPG count. Small random noise was added to alleviate overplotting. Lines represent the corresponding locally weighed scatterplot smoothing (LOWESS) curves.

With the estimation of local maxima we found a total of 65 local maxima in the 26 individuals with 6 or more stool examinations before treatment, which corresponds exactly to the expected number of local maxima in randomly generated numbers (median 65, 95% CI: 60-71) indicating no cyclic larvae release.

10.4.3 Larval excretion pattern after ivermectin treatment

On the first day of follow-up after treatment, 16 (33.3%) participants provided a stool sample, 9 (56.3%) were found positive (Figure 10.3). On the second day, 18 (37.5%) provided a sample, of which 4 (22.2%) still tested positive. On the third day, none of the 15 (31.3%) samples provided yielded any *S. stercoralis*. From day 3 to 21 days after treatment, on average 17 samples (35.4%) were returned each day, with a range from 11 to 24 samples per day. A total of 310 stool samples were received and analyzed with Baermann and Koga Agar culture. Not a single larva was detected.

From all the samples provided post-treatment we calculated the larvae reduction rate (LRR). The LRR increased dramatically to 92% and 99%, 24 and 48 hours after treatment, respectively (Figure 10.4). From the third day onwards all stool examinations remained negative.
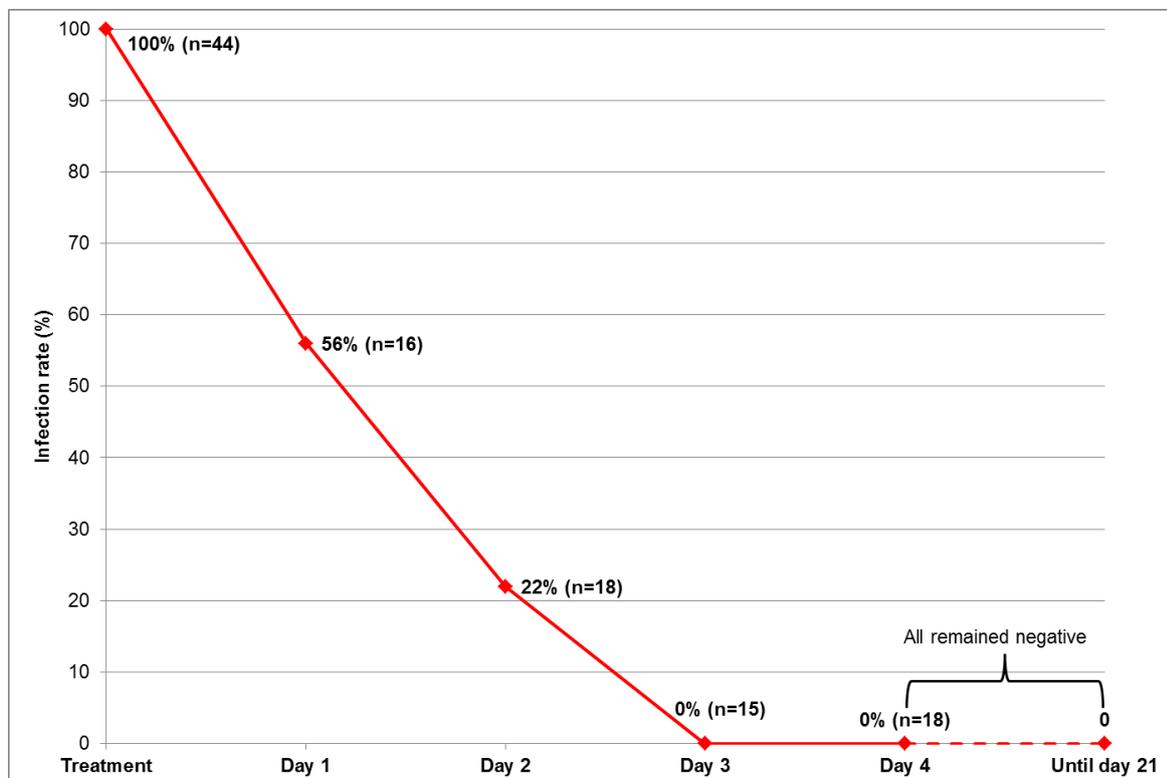
Figure 10.3 – *S. stercoralis* infection rate after ivermectin treatment
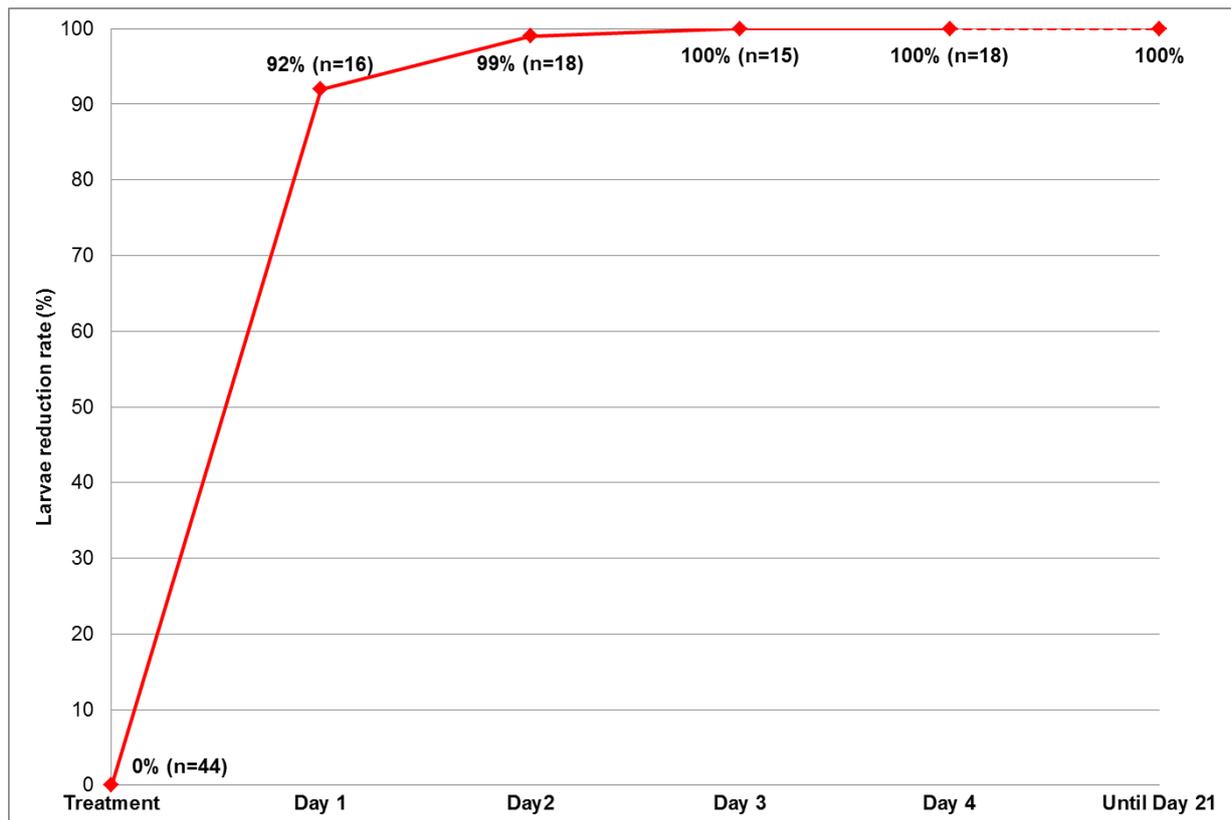
Figure 10.4 – Larvae reduction rate after ivermectin treatment

10.5 Discussion

S. stercoralis remains one of the most neglected und under-reported helminthic infection (Olsen et al. 2009; Schär et al. 2013b). The irregular excretion of larvae, as well as the very low sensitivity of standard O&P examination-procedures for *S. stercoralis*, requiring time-consuming special methods, are main factors contributing to this under-reporting. Our results show that there is considerable variation in the numbers of *S. stercoralis* larvae excreted. Individuals with high intensity infections (more than 10 larvae per gram stool) tend to present a positive result on all days of examination. Uparanukraw and colleagues (Uparanukraw et al. 1999) also documented variation of larval excretion, yet the underlying mechanisms remain unclear (Jones 1950; Jones and Abadie 1954; Leighton and MacSween 1990). As expected, we showed that the diagnostic sensitivity increases with higher infection intensity. The major challenge for diagnosis remains the low-intensity chronic infections, likely to be missed by a single examination.

To further increase diagnostic sensitivity and to minimize false-negatives, it is important to analyze multiple stool samples on consecutive days. We demonstrated that sensitivity

increases substantially if two samples are analyzed. For several other helminthic infections, at least three samples on consecutive days should be examined as has been recommended by others (Dreyer et al. 1996; Knopp et al. 2008; Marti and Koella 1993). For *S. stercoralis* it is essential to target eradication of the pathogen rather than reducing infection intensity, as the ability of auto-infection can sustain the infection in the host. Grove reported patients testing positive after two years even if the initial follow-up after treatment was not showing an *S. stercoralis* infection (Grove 1982).

When comparing the local maxima of larvae excreted to the maxima of random numbers, we could not establish a difference, which indicates that there is no cyclic pattern underlying the excretion of larvae. Therefore, when comparing the sensitivities of analyzing two stool samples on consecutive days with the sensitivity of analyzing two stool samples with one sampling free day in between, the sensitivities should be approximately the same. This is reflected in our results, the difference in the estimated sensitivities of two samples with a sampling free day in-between (97% vs. 99%) is likely due to chance. We assume that this small difference is due to the low-intensity infections, of which some had two consecutive days with no larvae excreted. Consequently one would expect a further increase in sensitivity when analyzing stool samples from 3 consecutive days.

The treatment with ivermectin proofed to be very effective. In all infected participants the excretion of larvae subsided. We investigated for the first time what happens directly after treatment. In our study all individuals that received treatment (200 μg/kg, single OD) stopped excreting larvae 72 hours after treatment by the latest. In none of the persons a larva could be detected during the 19 days of follow-up. However, the absence of larvae does not ensure that the parasite has been eradicated from the host, and a few surviving larvae may lead parthenogenetically again to high infection densities (Requena-Mendez et al. 2013; Satoh and Kokaze 2004). However, current diagnostic parasitological methods do not allow to verify this and prolonging the follow-up period is not an option in endemic areas, furthermore as the possibility of re-infection still will exist.

The standard Baermann method does not quantify the infection and only reports the presence/absence of the pathogen. We have modified the Baermann method for the calculation of larvae per gram stool. Counting larvae in a Baermann examination can be challenging especially in heavy infections with many larvae present. As the larvae are alive and moving, there is a possibility to miss or double-count larvae. The counts might therefore slightly differ from the true number, yet this difference is likely to be small and the calculated LPG-values are reflecting the intensity of infection appropiately.

Compliance is an issue in these studies, as very often participants are not able to defecate every day. It is very important to ensure that the stool samples provided are from the same person. If the person was not able to defecate, the stool container was returned empty. Furthermore it was important that the stool samples provided were fresh to ensure that larvae were still alive.

For studies not only focusing on other soil-transmitted helminthes but also *S. stercoralis*, the application of the best available coprological diagnostic methods like the Baermann and/or Koga Agar culture should be applied. This combination of diagnostic methods, although work- and material-intensive, would help to get more accurate numbers on the global prevalence situation of *S. stercoralis*. Today, most studies on helminthes do either not include *S. stercoralis* or use unsuitable diagnostic approaches, which, due to the factors stated above, leads to a severe underreporting of *S. stercoralis* world-wide (Schär et al. 2013b).

In our study we could show the importance of examining multiple stool samples on consecutive days because of the irregular excretion of larvae, and the danger of missing low-intensity infections. Furthermore we showed that treatment with ivermectin leads to the eradication of larvae excreted within three days after treatment. This has implications for the planning and conducting of follow-up studies. Our data indicate that the clearance of larvae is rapid. Even if a possible biological variability in excretion is taken into account, follow-up periods in ivermectin trials can be short, i.e. four days.

10.6 Acknowledgements

10.7 Author contributions

FS, HPM and PO conceived and designed the study; FS and VK collected data under the supervision of SM; FS and HPM coordinated the field laboratory activities; VK, MCC and SM coordinated the field work in Cambodia; JH, FS, HPM and PO analyzed data and interpreted the results; FS wrote the manuscript with PO; PO supervised the first author in all aspect of the study; all authors have read and approved the final version of manuscript.

10.8 Financial support and conflict of interest

11. *Strongyloides stercoralis* genotypes in humans in Cambodia

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Highlights:

We found three different SSU genotypes of *S. stercoralis* in humans

The three genotypes co-exist in the same individuals

No heterozygous worms were found, indicating no or minimal interbreeding

Graphical abstract

Hyper variable region I

tttatttgattaaaccatttttatattggttgactcaaaata

160   170   180   190

4T/5T
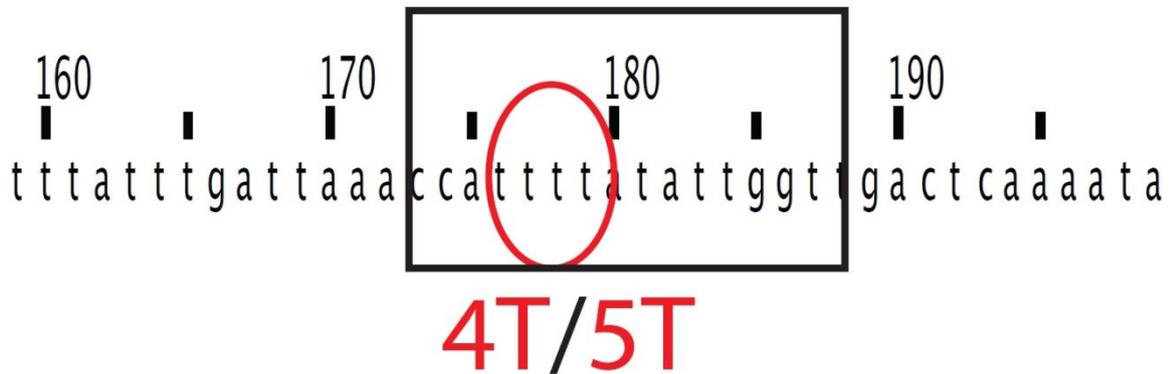
11.1 Abstract

Little is known about the genetic variability of the soil-transmitted nematode, *Strongyloides stercoralis,* in humans. We sequenced portions of the small subunit rDNA (SSU), including the hyper variable regions (HVR) I and IV from *S. stercoralis* larvae derived from individuals living in a rural setting in Cambodia. We identified three polymorphic positions, including a previously reported one within the HVR I. HVR IV was invariable. Six different SSU alleles existed in our sample. Although different genotypes of *S. stercoralis* were found in the same individuals, no heterozygous larvae were found. This indicates that there is no or very little interbreeding between the different genotypes. Further studies are needed to examine if this is because sexual reproduction, which is facultative, is rare in our study area's *S. stercoralis* population or because what is considered to be *S. stercoralis* today is actually a complex of closely related species or subspecies.

Keywords: *Strongyloides stercoralis*, SSU hyper variable region I / IV, genotype, human, Cambodia

11.2 Introduction

Strongyloides stercoralis is one of the most neglected helminth infections among the so-called neglected tropical diseases (NTDs) (Olsen et al. 2009). This soil-transmitted nematode is found world-wide (Bethony et al. 2006; Schär et al. 2013b), with the highest concentrations being in the tropical regions of Africa, South-East Asia and Latin America (Schär et al. 2013b). The parasitic adults are all parthenogenetically reproducing females that give rise to infective (parasitic) female progeny (homogonic cycle) and progeny that form a facultative free-living generation of male and female adults (heterogonic cycle) (Genta 1989; Grove 1996; Iriemenam et al. 2010). A suitable host is infected when filariform larvae penetrate the skin. Humans may also carry *Strongyloides fuelleborni* infections in Africa and Papua New Guinea (Genta 1989; Grove 1996; Liu and Weller 1993).

The small ribosomal sub-unit (SSU) rDNA sequence is highly conserved. In nematodes, it is frequently used for phylogenetic studies among and within species (Blaxter et al. 1998; Floyd et al. 2002; Hasegawa et al. 2009; Herrmann et al. 2006). In the genus *Strongyloides,* SSU sequences are useful markers for molecular taxonomy (Dorris et al. 2002; Eberhardt et al. 2008; Hasegawa et al. 2009; Hasegawa et al. 2010). A few studies on within-species variations in the SSU and in mitochondrial DNA sequences have been conducted on *S. stercoralis* originating from different locations and hosts (Hasegawa et al. 2009; Hasegawa et al. 2010; Hu et al. 2003; Koosha et al. 2009; Pakdee et al. 2012). Nevertheless, information on genetic variation in *S. stercoralis* is still scarce. In particular, we are not aware on any studies of the genetic structure of *S. stercoralis* populations within a particular location.

We examined the SSU genotypes of *S. stercoralis* larvae from humans in highly *S. stercoralis* endemic villages in Cambodia and report on the presence of different co-existing but not regularly interbreeding *S. stercoralis* genetic strains.

11.3 Methods

11.3.1 Study area and design

The study was part of a cross-sectional survey of intestinal helminth infections conducted in randomly selected villages in Preah Vihear and Takeo provinces in Cambodia, in March 2010 and 2011, respectively. Two stool samples were collected from members of selected

households on two consecutive days and examined for intestinal helminth infections. If *S. stercoralis* was diagnosed, larvae were collected and fixed in 70% ethanol for genotyping.

11.3.2 Field and laboratory procedures

Stool samples were collected each morning and analysed in the laboratory within three hours. A Baermann test (Baermann 1917) was performed and *S. stercoralis* larvae were collected for genotyping. In brief, stool was placed in a funnel fitted with a wire mesh and gauze. The funnel was filled with tap-water while a clamp keeps the water in the funnel. Artificial light was placed in front of the funnels to animate larvae to move out of the stool. After 2 h, the clamp was released and the water containing the larvae was collected in a tube. After centrifugation at 2000 rounds per minute (rpm), the effluent was examined for the presence of *S. stercoralis* larvae. If larvae were present on the first slide, the sediment was conserved with 70% ethanol.

11.3.3 Genotyping single *S. stercoralis* larvae

We sequenced about 450 bp covering the hyper variable region (HVR) I (c.f.(Hasegawa et al. 2009)) from 269 individual *S. stercoralis* larvae. This region had been used for molecular taxonomy in multiple studies of *Strongyloides* and other nematodes (Blaxter et al. 1998; Dorris et al. 2002; Eberhardt et al. 2008; Floyd et al. 2002). It is frequently invariable within nematode species (Herrmann et al. 2006). For *S. stercoralis*, a within-species polymorphism has been reported (Hasegawa et al. 2009). For 151 of our larvae, we also sequenced a fragment containing HVR IV, which is known to vary between, but not normally within, *Strongyloides* species (Hasegawa et al. 2009). The exact procedures were as follows: Single larvae were prepared for polymerase chain reaction (PCR) amplification, as described by Eberhardt and colleagues (Eberhardt et al. 2007). In brief, single larvae were picked out and incubated in 10 µl 2 x lysis buffer (20 mM Tris-HCl pH 8.3, 100 mM KCl, 5 mM MgCl2, 0.9 % NP-40, 0.9 % Tween 20, 0.02 % Gelatine, 240 µg/ml Proteinase K) at 65 ºC for 2 h, followed by 95 ºC for 15 min. The HVR I region was amplified using primers, RH5401 (5'-AAAGATTAAGCCATGCATG-3') and RH5402 (5'-CATTCTTGGCAAATGCTTTCG-3') and sequenced with RH5403 (5'-AGCTGGAATTACCGCGGCTG-3'), as described by Eberhardt and colleagues (Eberhardt et al. 2007). The HVR IV region was amplified with primers, 18SP4F (5'-GCGAAAGCATTTGCCAA-3') and 18SPCR (5'-ACGGGCGGTGTGTRC-3') as described by Hasegawa and colleagues (Hasegawa et al. 2009) and sequenced using the same primers.

11.3.4 Ethics Statement

The study was approved by the National Ethics Committee for Health Research (NECHR), Ministry of Health, Cambodia and the ethics committee of the Cantons of Basel-Stadt and Basel-Land (EKBB), Switzerland. All participants were informed of the study aims and procedures and provided written informed consent prior to enrolment. All data handled remained strictly confidential. All individuals infected with *S. stercoralis* were treated with ivermectin (200 µg/kg BW) over two days.

11.4 Results and Discussion

We sequenced the region around HVR I from 269 *Strongyloides* larvae isolated from 29 different people (Figure 11.1) and compared the sequences with each other; with the sequence with accession number AF279916, reported by Dorris and colleagues (Dorris et al. 2002); and with the three sequences with accession numbers AB453314, AB453315, and AB453316, reported by Hasegawa and colleagues (Hasegawa et al. 2009) (Figure 11.2). All our sequences differed at two positions from AF279916. Position 27 was T, not G and starting at position 38 there were three As not four. The sequences reported by Hasegawa and colleagues (Hasegawa et al. 2009) do not cover these positions.

Furthermore, among our samples, we found two polymorphic positions. First, the stretch of Ts starting at position 176 consisted of either 4Ts or 5Ts (Figure 11.2). This is the same polymorphism Hasegawa and colleagues reported earlier (Hasegawa et al. 2009). Second, at position 458 we found either a T or an A (Figure 11.2). At this position all three sequences from Hasegawa and colleagues had an A and differed from AF279916, which has T.

Of the four possible combinations, three (accession numbers KF926658-KF926660) existed in our samples, namely 4T+T (read four Ts at position 176 and T at position 458) (like AF279916; in Fig. 1 represented by I in a yellow cell), 5T+T (not previously described; in Figure 11.1 represented by II in a red cell) and 5T+A (like AB453315; in Figure 11.1 represented by III in a blue cell). No worms heterozygous for any two of these versions were found. Since studies on other nematodes found this region to be virtually invariable within a particular species (Eberhardt et al. 2008; Floyd et al. 2002; Mayer et al. 2007), it was surprising that we found two polymorphisms. Therefore, for a fraction of our samples (Figure 11.1), we also determined the sequence of the HVR IV. Hasegawa and colleagues (Hasegawa et al. 2009) found the HVR IV to be invariable among the different isolates of *S stercoralis* analysed and, at the same time, to be quite different between most species. All 151 HVR IV sequences were identical to the previously reported sequence for *S. stercoralis.*

However, we did identify a polymorphic position somewhat outside of HVR IV. Position 1454 was either A or G (accession numbers KF926662 and KF926661, respectively). AF279916 has an A in this position, while AB453314, AB453315, AB453316 do not cover this nucleotide.

Village	SV				VP	PS	TK	KT	TC		CH	KG	VT	TR	PH	
Household	6	9	11	16	9	9	0	4	1	6	7	5	11	8	2	13
Individual	1	1	1	2	4	2	4	3	2	3	2	3	3	5	3	4
Genotypes	I A	II G	II G	I	II G	II G	II G	II G	I G	II A	II G	II G	I G	III A	II A	II G
	I A	II G	II G	II G	II G	II G	II G	II G	I G	II A	II A	II G	I G	III A	II	II G
	I A	II G	II G	II G	II G	II G	II	II G	I	II A	II A	II G	I G	III A		II G
	I A	II G	II G	II A	II G	II G	II	II G		II A	II A	II G	I G	III A		II
	I A	II G	II G	II A	II G	II G	II	II A		II A	II A	II G	I G	III A		II
	I	II G	II G	II A	II A	II G	II	II A		II	II A	II A	I G	III A		II
	I	II G	II G	II	II A	II G	II	II		II	II A	II A	I G	III A		II
	I	II G	II G	II	II	II	II	II		II	II A	II A	I A	III A		II
	I	II G	II G	II	II	II	II				II A	II A	I A	III A		II
	I	II G	II G	II	II	II	II				II A	II A	I A	III A		II
	I		II G	II	II		II				II A	II	I	III		II
			II G	II	II		II				II A	II	I	III		II
											II A					

Village	CS						PR			TD			
Household	1	1	7		10	14	9	11		14	10		
Individual	1	2	1	3	1	1	2	2	1	4	2	2	4
Genotypes	II	II	II	I	II G	II	III G	I A	II	II A	I G	II G	II A
	II		II	I	II G	II	II A		II	II A	I G	II G	II A
			II	I	II G		II A		II	II A	I G	II G	II A
			II	I	II G		II A		II	II A	I G	II G	II
			II	I	II A		II A		II	II A	I G	II A	II
			II	I	II A		II A		II	II A	I G	II A	II
			II	I	II		II A		II	II A	I A	II A	II
			II		II		II		II	II A	I	II A	II
			II				II		II	II	I	II	II
									II		I		II
									II				
									II				
									II				
									II				
									II				
									II				
									II				
									II				

Figure 11.1 – SSU genotypes of *S. stercoralis* worms isolated from individual persons. Each cell corresponds to one worm, worms isolated from one patient are in the same column. The three different variants of the region around HVR I are indicated as follows:

I: positions 176-179 = 4 T; position 458 = T (KF926658)

II: positions 176-179 = 5 T; position 458 = T (KF926659)

III: positions 176-179 = 5 T; position 458 = A (KF926660)

The GenBank accession numbers are given in (). If position 1454 was determined the nucleotide (A or G) is given after the roman numeral (accession numbers KF926662 and KF926661 respectively)

Figure 11.2 – *S. stercoralis* 18S SSU (AF279916) used as reference sequence. Positions found to be polymorphic in human samples are indicated with a star*. Positions that were not polymorphic in our samples but differed from the reference sequence are marked with a plus +. Hyper variable regions I and IV are boxed.

When all three polymorphic sites described here were combined, we found six different SSU alleles in worms isolated from humans that one would consider as *S. stercoralis*. At least 93 worms were isolated from hosts that contained worms of multiple genotypes (for another 22 individuals, worms with different genotypes were isolated from the same household) demonstrating that the different genotypes co-exist sympatrically, thus there should be ample opportunity for crossing. Nevertheless, we did not identify a single heterozygous worm. This suggests that the carriers of the different SSU alleles in our study area interbreed only very rarely, if at all. An obvious explanation for this would be that the different SSU alleles represent separate populations of interbreeding animals and would have to be considered separate species. However, at the moment, we have no proof for within group sexual reproduction. It is also possible that sexual reproduction is rare or absent in at least certain lines of *S. stercoralis* in our study area. This could be either because successful reproduction occurs exclusively through the parthenogentic direct cycle or because reproduction in the free-living generation is non-sexual, in spite of the existence of males. Indeed, based on cytological studies, it was proposed that reproduction in the free-living generations of multiple species of *Strongyloides* is by sperm dependent parthenogenesis (pseudogamy) (for *S. ratti* (Nigon and Roman 1952); for *S. papillosus* (Triantaphyllou and Moncol 1977); for *S. stercoralis* (Hammond and Robinson 1994)). Using molecular genetic approaches, this hypothesis was disproved and sexual reproduction has been demonstrated for *S. ratti* (Viney et al. 1993) and for *S. papillosus* (Eberhardt et al. 2007). To our knowledge, no genetic investigation has been conducted with *S. stercoralis* and pseudogamic reproduction remains a possibility in this species. Nevertheless, although, based only on this study, it is too early to draw any firm conclusions, one should consider the possibility that what is traditionally considered to be *S. stercoralis* is actually a complex of closely related species or subspecies.

11.5 Acknowledgments

11.6 Authors' contributions

FS, LG, HM, AS and PO designed the study; FS and VK collected samples in 2010; FS collected samples in 2011 and conducted field laboratory analysis; SM had the overall responsibility of data collection; LG and AS conducted the genotyping of the larvae, LG and FS analysed data and interpreted results together with AS, HM and PO; FS and PO wrote

the manuscript; AS, LG, SM and HM assisted with manuscript revisions; all authors read and approved the final submitted manuscript; AS and PO are guarantors.

11.7 Financial Support

12. General discussion and conclusions

This work was jointly undertaken in collaboration with the National Center for Parasitology, Entomology and Malaria Control, Ministry of Health, Phnom Penh, Cambodia and the Swiss Tropical and Public Health Institute, Basel, Switzerland. The generous funding of the UBS Optimus Foundation made it possible to set up a large scale project targeting the neglected nematode *S. stercoralis*. In the framework of this collaboration a comprehensive set of studies was conducted on *S. stercoralis* and co-infections, epidemiology, morbidity and treatment. A first step was the summary of today's information on *S. stercoralis* at the global level. We conducted an extensive literature research, including all information on the prevalence, risk factors and treatment. The second part of our work included the study of epidemiological determinants of *S. stercoralis* at household level in an endemic setting, namely in Cambodia. We conducted community-based surveys, assessing the prevalence of *S. stercoralis* and co-infections. We examined the humans and animals of the same villages to be able to draw conclusions about transmission dynamics. A third part consisted of detailed analysis of *S. stercoralis* and strongyloidiasis at the individual level. We genotyped single worms from humans and animals. We further assessed the excretion dynamics of larvae in infected individuals and the impact of treatment. Additionally, we tested and evaluated molecular diagnostic methods for its applicability in a field setting.

12.1 Prevalence, diagnosis, treatment and risk factors for *S. stercoralis:* the global picture

The first main part of this work consisted of collecting, summarizing and analyzing today's scientific information about *S. stercoralis*. We have performed a literature review, searching all available literature and data on the prevalence and the treatment of *S. stercoralis*. The rationale behind this review stems from the lack of knowledge about the prevalence that exists today. The last reviews were undertaken in 1989 by Genta (Genta 1989), some 25 years ago, and Jorgensen in 1996 (Jorgensen et al. 1996). Today, most of the scientific literature reports the estimates for the global prevalence of *S. stercoralis* to be between 30-100 million people infected world-wide, although there is agreement that these numbers are most likely to be an underreporting of what the true prevalence is (Olsen et al. 2009).

12.1.1 Prevalence

In our review we could include 354 studies that reported prevalence data on *S. stercoralis*. Importantly, we could show that most studies did not focus on *S. stercoralis* but on other STH-infections. This is especially true in community-based studies, which comprised more

than 50% of all studies included. There are only two countries worldwide, namely Brazil (43 studies) and Thailand (40 studies), that reported a substantial number of studies. We demonstrated that many countries that are considered *S. stercoralis*-endemic or have the ideal ecological and socio-economic conditions for the presence of *S. stercoralis* have no or only little data on the prevalence. This can be best demonstrated with the absence of studies in countries in sub-Saharan Africa, where we could identify studies from only 20 (43.5%) of the 46 countries from the WHO-African region.

Our work aimed at contributing to the general understanding of the distribution of *S. stercoralis*. The prevalence data collected from our literature search was used to establish estimations of the prevalence for each country. The collected data was not only scarce compared to other STHs, it was also very heterogenous and divers, especially concerning the diagnostic methods applied. For the mapping of the prevalence, we had to summarize the available data. This is always a trade-off, as the summary makes the data become more generalized and less detailed. The plotting of the maps was done on a country-scale. When looking at the maps, one has to keep in mind that the data is often only derived from a few studies. In big countries, with many different ecological settings, with socio-economic differences and differences between rural and urban populations, it is clear that the true prevalence rates are likely to be diverse as well and the reported prevalence rates are not applicable for all parts of the country. Furthermore, studies are often performed in high risk areas for STH-transmission. Extrapolating results from these specific high risk parts of a country to the whole country can lead to an overestimation of the prevalence. Yet, the summary on country-level is a good way of collapsing the data available and use the results produced as the backbone to demonstrate further research needs.

Generally, the absence of reliable data on the prevalence of *S. stercoralis* today is based largely on the exclusion from surveys on helminthic infections. These surveys still largely focus on the three so-called major STHs, namely *A. lumbricoides*, *T. trichiura* and the hookworms (Krolewiecki et al. 2013). There are control programs aiming at these three STHs in place, mainly focused on school-aged children, which have the highest burden to carry from STHs infections (Albonico et al. 2008). Yet, the lack of information available for *S. stercoralis* prevalence also leads to the exclusion of this parasite from control programs.

12.1.2 Diagnosis

Our review demonstrated that the main factor leading to the underreporting of *S. stercoralis* prevalence is the diagnosis. Strikingly, from all the studies included, almost two-third (63.3%) applied diagnostic methods showing a very low sensitivity for the detection of *S. stercoralis*. In the community-based surveys, the best coprological methods are only applied in around

30% of the studies, and almost never in combination. While serological and molecular diagnostic methods do not even constitute 10% of the applied diagnostic methods in community- and hospital-based studies, they make up more than 20% in studies on refugees and immigrants. These studies are most often undertaken in more developed countries that have the laboratory facilities and financial backing needed to perform these diagnostic tests.

We demonstrated that the reason for the underreporting is the use of inappropriate diagnostic methods. This, again, is due to the fact that *S. stercoralis* is not the focus of most studies on STHs. The Kato Katz method, the diagnostic method most commonly applied in surveys on STHs, is not applicable for the detection of *S. stercoralis* (Schär et al. 2013b). Other commonly applied coprological diagnostic methods such as the direct smear (Beaver et al. 1984), the FLOTAC (Knopp et al. 2014) and concentration methods such as the formalin-ethyl acetate concentration method (FECT) (Ritchie 1948) all show very low sensitivities. The Harada-Mori filter paper technique has a better sensitivity (Harada 1951). The most sensitive coprological methods today are the Baermann method (BM) (Baermann 1917; Lima and Delgado 1961) and the Koga Agar plate culture (KAP) method, whose applicability and good sensitivity has been demonstrated in multiple studies (de Kaminsky 1993; Khieu et al. 2013a; Knopp et al. 2008; Koga et al. 1990; Koga et al. 1991; Mahmoud 1996; Sato et al. 1995b; Sukhavat et al. 1994; van der Feltz et al. 1999) They are considered the best coprological methods available (Arakaki et al. 1990).

When comparing the BM and KAP, we found the following points. The BM has the advantage that it yields a result around 2-3 hours after the test has been set up, whereas the KAP method has a delay of at least two days, in which the agar plate with the stool is incubated. This can have implications for the planning in field studies, especially for treatment of infected participants. Furthermore does the BM require less material and less sophisticated equipment, and is therefore less costly (Graeff-Teixeira et al. 1997; Hernandez-Chavarria and Avendano 2001). Yet, the whole construction of the BM is more cumbersome and difficult to handle than the KAP method. Both methods have to be carefully executed, as both involve preparation-steps in which there is the danger of acquiring an infection as there are infectious larvae present (de Kaminsky 1993; Koga et al. 1990). In general, both methods are also seen to be laborious and time-consuming, another factor contributing to the absence in routine laboratory measures (de Kaminsky 1993). Both methods proofed their applicability, although there is a trend towards the KAP method, as it is considered superior to the BM by many authors (Grove 1996; Kobayashi et al. 1996; Koga et al. 1990; Marchi Blatt and Cantos 2003; Salazar et al. 1995; Sato et al. 1995b).

Additionally, the KAP is also able to detect hookworm infection if present. The eggs of hookworm can hatch during the two day period of incubation. It is therefore necessary to

distinguish between larvae of *S. stercoralis* and hookworm larvae. This is a crucial point and requires skilled microscopists (Liu and Weller 1993).

To summarize, it would be desirable to have a combination of the BM and the KAP method applied, as we have applied in our field studies in Cambodia (Khieu et al. 2013a; Khieu et al. 2013b; Schär et al. 2014b). Yet, our review demonstrated that this combination is hardly ever applied in large-scale community-based surveys. A good example is sub-Saharan Africa, where only 19.0% of the studies were conducted with either the BM or the KAP method (Schär et al. 2013b).

The challenges in diagnosing *S. stercoralis* infection with coprological methods called for serological diagnostic methods. Over the years, several immunodiagnostic assays have been developed and tested, most prominently immunofluorescence antibody tests (IFAT) (Bisoffi et al. 2014; Boscolo et al. 2007; Koosha et al. 2004) and the enzyme-linked immunosorbent assay (ELISA) (Bisoffi et al. 2014; Huaman et al. 2003; Koosha et al. 2004; Lindo et al. 1994; Sato et al. 1990; Schaffel et al. 2001; Sithithaworn et al. 2005; Sithithaworn et al. 2003; van Doorn et al. 2007).

The major challenges of ELISA are the need for production of antigens and the possible cross-reactivity with other helminth infections (Gam et al. 1987; Lindo et al. 1996). Latter may lead to an overestimation of the prevalence as well as the sensitivity, especially in areas were helminth multiparasitism is highly abundant (Genta 1989; Loutfy et al. 2002; Siddiqui and Berk 2001), which is the case in most endemic countries. Recently, ELISA assays relying on recombinant antigens that can easily be purified (Krolewiecki et al. 2010; Ravi et al. 2002) and show negligible cross-reactivity with other helminth infections (Ramanathan et al. 2008) have been developed and evaluated.

In our review we could identify the absence of high sensitivity diagnostic methods especially in large-scale community-based studies. As stated above, only 10% of our studies included applied serological diagnostic methods. Most were at sophisticated laboratories (i.e. screening of refugees in the US, Australia etc.). We argue that this is due to better laboratory infrastructure, better financial situation and the most often smaller numbers of individuals that have to be examined.

12.1.3 Treatment

We have performed a second review, focusing on the treatment of *S. stercoralis*. The drugs that are reported to have the best efficacy for strongylodiasis treatment are Ivermectin and Thiabendazole. Ivermectin is considered the drug of choice today, as it is as effective as Thiabendazole, while having less and less severe side-effects (Bisoffi et al. 2011).

We could identify 68 studies, including 14 different substances that reported efficacy of treatment for *S. stercoralis*. The most common drugs tested were Ivermectin (in 30 studies), Albendazole (in 23 studies), Thiabendazole (in 21 studies) and Mebendazole (in 5 studies). The results of our meta-analysis confirmed the superiority of Ivermectin and Thiabendazole compared to the other drugs. Both had a very high pooled cure rate with 0.97 and 0.95, respectively. Additionally, we identified 10 further substances used, which have only been studied on a small-scale. To evaluate them as future potential drug candidates, further detailed studies would be needed.

The standard treatment regimen for Ivermectin is a single dose of 200 µg/kg bodyweight, administered orally We evaluated the effect of single dose Ivermectin treatment in our field study on the excretion of larvae (Schär et al. 2014b). We conducted a follow-up of 21 days after treatment, analyzing a stool sample each day. All 44 treated participants stopped excreting larvae after three days, and remained negative, indicating a cure rate of 100%. Our diagnostic control included two coprological methods, the BM and KAP. The study by Bisoffi and colleagues compared Ivermectin with Thiabendazole. For Ivermectin they used the single dose approach and established a cure rate of 85.7% when control was conducted with coprological diagnostic methods. The efficacy dropped to 56.6% if a negative IFAT result or the decrease of two or more antibody titres was defined as "cured" (Bisoffi et al. 2011). We do not know how the inclusion of serological methods such as the IFAT would have affected our cure rate. In general, it is difficult to establish the true cure rates for any treatment for strongyloidiasis in endemic settings, as there is the possibility of re-infection. For further evaluating the cure rate of Ivermectin and the effect of different treatment regimes, studies on proven cases of strongyloidiasis in non-endemic settings would be ideal, as this would rule out re-infection.

Additionally, with the quantification of the BM method, we could establish a larvae reduction rate (LRR) after treatment, similarly to the egg reduction rate (ERR) applied for other helminthic infections. The LRR can have implications for the assessment of treatment success, although for *S. stercoralis*, unlike with other helminth infections, total eradication of the parasite from the host is the target for treatment. Nevertheless, future studies should establish possible associations between the intensity of infection and the acute morbidity caused in the host and whether the LRR can be seen as a proxy of decrease of burden.

In preventive mass-treatment, the treatment relies on single dose treatments. It therefore is essential to establish what the efficacy of a single dose Ivermectin is. In our meta-analysis, we could demonstrate that the 200µg/kg single dose had a cure rate of 0.95. When the single dose was administered on two days, the cure rate is raised to 0.98, the best cure rate observed for any treatment and regimen. As for the mass treatment, a single dose is

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preferred, it would be interesting to see, if the doubling of the total drug dose would yield the same result when administered on a single day.

The drugs that are recommended for the treatment of STHs include Albendazole, Medendazole, Levamisole and pyrantel/oxantel. (Albonico et al. 2006). For Levamisole and pyrantel/oxantel, we could not identify any studies. For Mebendazole and Albendazole we report a pooled cure rate of 0.84 and 0.81, respectively. Although showing moderate efficacy, we argue that both drugs cannot be seen as an alternative treatment, because in the treatment of *S. stercoralis*, unlike with other STHs, treatment success is seen as the total eradication of the parasite from the host and not only reducing the worm burden (Grove 1996; Krolewiecki et al. 2013)

As stated before, Ivermectin should be the drug of choice for the treatment of strongylodiasis today. An advantage is that Ivermectin has been included into nationwide mass drug administration (MDA) programs for combating lymphatic filariasis and therefore solid evidence exists on the safety and applicability in mass treatment programs (Rebollo and Bockarie 2013). Inclusion of Ivermectin in MDA-programs aimed at STHs seems to be an option in highly endemic areas.

Extensive use of drugs can increase the development of resistances. Although, there have been no resistances reported for Ivermectin in humans so far, resistances have been reported in veterinary medicine, where Ivermectin has been used originally (Fox 2006). New alternative drugs are tested today, most prominently Tribendimidine. The drug has a broad spectrum against helminthic infections (Xiao et al. 2005) and its activity has been proven in the laboratory on *S. ratti* (Keiser et al. 2008) First studies have been conducted in humans in China, with an observed cure rate of 54.5% (Steinmann et al. 2008). Our literature research showed other drug candidates, for instance Mirazid. Yet the data obtained is based on small-scale studies. To further establish new drug candidates, their effect on *S. stercoralis* needs to be demonstrated in in vivo or in vitro laboratory studies.

12.1.4 Risk factors for *S. stercoralis*

There are some well-established risk factors for infection with *S. stercoralis*. They include personal behavior such as walking bare-footed and bad hygiene. Other contributors are bad sanitary conditions and the use of night-soil as fertilizer, as this promotes the spread of infective larvae in the environment (Khieu et al. 2013a; Ziegelbauer et al. 2012).

In our review we focused on risk factors and associations between specific health conditions in combination with *S. stercoralis* infection. This included infection with HIV and HTLV-1, as well as immunosuppression caused by treatment, conditions which can cause immune

suppression and therefore lead to the most severe outcome of *S. stercoralis* infection: disseminated strongyloidiasis and hyperinfection.

In our review, we could identify 16 studies that compared HIV-positive individuals with sero-negative controls for infection status. Our meta-analysis revealed a significantly higher risk for the HIV-infected individuals to be concomitantly infected with *S. stercoralis* (OR: 2.17 (95% BCI: 1.18 – 4.01)). This reported odds ratio, although significant, is comparably small, which agrees with the case-control studies included, as only three reported a significant difference between HIV-positive and sero-negative controls (Feitosa et al. 2001; Gomez Morales et al. 1995; Pinlaor et al. 2005) and a further three reported a similar or reduced risk (Dias et al. 1992; Fontanet et al. 2000; Lebbad et al. 2001; Mendez et al. 1994).

Interestingly, despite the higher risk for infection, disseminated strongyloidiasis or hyperinfection in HIV-positive individuals is relatively rare. Only a few cases are reported in the literature (Keiser and Nutman 2004). In HIV-infected individuals, the immune system is altered and suppressed, which lead to the assumption it would favour the development of disseminated strongyloidiasis and therefore *S. stercoralis* should be a "marker infection" for advancing HIV disease (Viney et al. 2004). Yet soon after the onset of the HIV-pandemic, this assumption was regarded to be untrue (Gompels et al. 1991; Lucas 1990). Until today, the reason for why disseminated strongyloidiasis is relatively rare in HIV-positive individuals is discussed. It is thought that through modulation of the immune system in HIV infected individuals and the change of CD4+ cells, the reduction of CD4+ cells leads to the promotion of indirect larval development. This could explain the absence of disseminated strongyloidiasis in advancing HIV disease (Viney et al. 2004). Additionally, in experimental studies it has been shown that IgG could be a protective factor against migrating larvae (Ligas et al. 2003; Murrell 1981). HIV-infection does not affect the IgG responses to *S. stercoralis* (Viney et al. 2004), and therefore also in advancing HIV-disease the IgG could act as protection against migrating larvae. Furthermore, the HI-virus modulates the immune system, increasing Th2 cytokines and decreasing Th1 cytokines (Barker et al. 1995; Tanaka et al. 1999; Valdez and Lederman 1997). In non-HIV-infected individuals, it is known that the infection with *S. stercoralis* leads to elevated IgE levels (Genta 1986), which are seen as an indicator for a Th2 immune response, known to occur in many helminthic infections (Finkelman et al. 1997). Therefore, the modulation of the immune response by the HI-Virus might lead to an immune response pattern that favors bacterial and viral opportunistic infections rather than helminthic infections (Concha et al. 2005) and might explain why HIV-positive individuals, also with advanced HIV-infection status, do not develop disseminated strongyloidiasis more often than non-HIV-positive individuals infected with *S. stercoralis*.

The human T-lymphotropic virus type 1 (HTLV-1) is a human retrovirus, which can cause T-cell leukaemia and lymphoma. It is largely restricted to some endemic areas that are distributed globally (Proietti et al. 2005). It has been shown that individuals infected HTLV-1 tend to be significantly more often co-infected with *S. stercoralis* in comparison with HTLV-1-seronegative controls (Chieffi et al. 2000; Courouble et al. 2004; Einsiedel and Woodman 2010; Hayashi et al. 1997; Marsh 1996; Nera et al. 1989; Phelps 1993; Robinson et al. 1994). Our meta-analysis showed an odds ratio for infection of 2.48, although it was not a significant result (95% BCI: 0.70 – 9.03). This might be due to the small number of studies (four) that we could include in the meta-analysis.

As in HIV-infection, the increase of the prevalence of disseminated strongyloidiasis and hyperinfection has been documented (Gotuzzo et al. 1999), yet the frequency with which it occurs is not well established.

Immunosuppressive treatment, mainly with glucocorticoids, can therefore be seen as one of the main risk factors for disseminated strongyloidiasis and hyperinfection (Keiser and Nutman 2004). It has been argued that the glucocorticoids can have a direct effect on *S. stercoralis*, either through the rejuvenation of reproductively latent adult females (Mansfield et al. 1996) or through an acceleration of the transformation of rhabditiform larvae to invasive filariform larvae (Harvey et al. 2000). This increase of filariform larvae promotes the hyperinfection (Genta 1992). Organ transplant recipients also can receive immune suppressive treatment, which in turn can lead to disseminated strongyloidiasis and hyperinfection. There are case reports, especially from kidney transplant recipients (DeVault et al. 1990; Ferreira et al. 2012; Morgan et al. 1986; Palau and Pankey 1997; Sadjadi et al. 2013). Additionally, it has recently been documented that recipients were infected with *S. stercoralis* through the kidney of the donor (Hamilton et al. 2011; Roseman et al. 2013).

12.2 Cambodia: *S. stercoralis* infection and co-infections

Our field work was conducted in Cambodia, located in Southeast Asia, a region where intestinal multiparasitism has been well documented (Chhakda et al. 2006; Khieu et al. 2013a; Sayasone et al. 2009; Sinuon et al. 2003). We aimed at assessing multiparasitism at household level with a focus on *S. stercoralis* in endemic rural villages in Preah Vihear province. These villages have the ideal ecological and socio-economic conditions for transmission of *S. stercoralis*. The climate is tropical, with alternating wet and dry seasons and high temperatures and humidity all year long. The sanitary conditions are poor and defecation often takes place in the open ("behind the house", forest, rice field etc.) which is a major source for the spread of larvae of *S. stercoralis* and eggs of other STHs and

subsequent infection of new hosts (Ziegelbauer et al. 2012). Further are most Cambodians living in rural villages relying on subsistence farming, often working in the rice fields. This work, combined with not wearing shoes, further exposes the villagers and makes infections easily possible.

Our study, which included 218 individuals, showed that multiparasitism is the norm rather than the exception. Altogether we could diagnose eight different helminth parasites and six protozoan parasites. Almost 90% of the participants were diagnosed with at least one infection. The highest number recorded was seven concomitant infections. The major parasites diagnosed in humans were hookworms (63.3%), *Entamoeba* spp. (27.1%), *S. stercoralis* (24.3%), *G. duodenalis* (22.0%) and *Blastocystis* (18.4%).

When assessing the prevalence rates in different age groups, we found that the prevalence of *S. stercoralis* is around 20% in children from 2 to 10 years, adolescents from 11 to 20 years and young adults from 21 to 30 years. Prevalence increases to around 30% in the age-group 31 to 50 years and is peaking at almost 35% in persons older than 51 years. These results are consistent with the findings of our large-scale study conducted in Preah Vihear. In 1071 *S. stercoralis* cases, the observed prevalence in children was 31.4% and rose to 51.4% in adults (Khieu V 2014). This pattern in the age-distribution could be because of the auto-infection. Persons only need to be infected once in their life, and without appropriate treatment, the infection can be sustained, up to 75 years (Concha et al. 2005; Genta 1992; Keiser and Nutman 2004; Prendki et al. 2011; Vadlamudi et al. 2006).

Interestingly, hookworm infections were almost three times more often diagnosed than *S. stercoralis*. Both nematodes have the same route of transmission. In our case, this difference might be due to the PCR analysis, which was only performed for hookworm infection. Indeed, the Kato Katz method only detected a prevalence of 21.1% for hookworm infection. Because of the same route of transmission, it was argued that hookworm infections can be seen as a proxy for the prevalence of *S. stercoralis* prevalence (Bisoffi et al. 2013). Yet because of the lack of data, it is hardly possible to establish a rate. Bisoffi and colleagues argue that *S. stercoralis* prevalence is most likely to be around ¼ up to same prevalence as hookworm. In our study, the prevalence rates detected (excluding the PCR results) is about the same. To know the ratio in more detail could help to compile better estimates of how many persons infected with *S. stercoralis* exist globally, based on the numbers estimated for hookworm infection, which are more accurate. The recently published study by Pullan and colleagues (Pullan et al. 2014) estimates that around 440 million people are infected with hookworm. This number could well be closer to the true prevalence of *S. stercoralis* than the often cited 30-100 million.

In developing countries, co-infections with several intestinal parasites are a common occurrence, most often in the poorest of the poor (Drake and Bundy 2001; Petney and Andrews 1998). Yet, many epidemiological surveys focus on one specific parasite and therefore, only one specific diagnostic method, the one most appropriate for the studied parasite, is applied. With this, only the parasites that are detected by the diagnostic method are reported, neglecting possible co-infections with parasites not detectable. It is therefore crucial to apply several diagnostic methods together to draw a more comprehensive picture of multiparasitism in a given study population. Keiser and colleagues stated that the absence of one diagnostic method detecting all intestinal parasites with a high sensitivity is lacking, hindering extensive studies on multiparasitism (Keiser et al. 2002). In the absence of such a diagnostic "golden bullet", the way forward remains the application of a combination of diagnostic methods.

In our study on intestinal parasitic infections, we have applied a wide range of diagnostic methods, including the KK, KAP, BM on two samples and the FECT, Flotation and PCR on one sample. This combination of diagnostic methods made it possible to assess intestinal parasitic infections in more detail. We could identify eight different helminth species and six different protozoan species. Only one tenth of participants were infection-free, and some participants harboured up to seven infections concurrently. All these examinations were performed on stool samples. Further insight could be gained if blood samples of each participant would be analysed as well.

12.2.1 Multiparasitism: zoonotic co-infections and the role of animals

When assessing multiparasitism in inhabitants of communities and implementing control, it can be an additional input to analyse the animals present in the communities as well. In our field study, we have collected stool from dogs and pigs that lived in the same households as the examined people. To ensure that only animals belonging to the household were included, we collected the stool directly from animals identified by the household owners with the help of a veterinarian. We could collect samples from 94 dogs and 76 pigs.

The most frequent infections found in dogs were hookworm (80.8%), *Spirometra* spp. (21.3%) and *Strongyloides* spp. (14.9%). More than 80% of the dogs had at least one infection, and multiple infections were observed in more than half (53.2%) of the dogs. Eleven species of parasites (eight helminths and three protozoa) were identified in dogs, seven of which have zoonotic potential (hookworms, *Strongyloides* spp., *Trichuris* spp., *Toxocara canis*, *Echinostoma* spp., *G. duodenalis and Entamoeba* spp.).

For the pigs, the most frequent infections found were *Isospora suis* (75.0%), *Oesophagostomum* spp. (73.7%) and *Entamoeba* spp. (31.6%). Of the 76 pigs, 74 (97.4%)

had at least one parasitic infection and 66 (86.8%) had multiple infections. Altogether, twelve different parasitic infections were identified (eight helminthic and four protozoan infections) five of which are known for their zoonotic potential (*Ascaris* spp., *Trichuris* spp., *Capillaria* spp., *Balantidium coli* and *Entamoeba* spp.).

For the hookworms collected in dogs and humans, we have performed a detailed molecular. It showed that 90% of the hookworm positive dogs were infected with *Ancylostoma ceylanicum*. In humans, it is generally reported that the two major species found are *Necator americanus* and *Ancylostoma duodenale*, in combination known as "hookworm" infection. The existence of *A. ceylanicum* in humans has been demonstrated in Southeast Asia, but the most frequent hookworm species is reportedly *N. americanus* (Conlan et al. 2012; Jiraanankul et al. 2011; Ngui et al. 2012; Traub et al. 2008). Yet, our molecular analysis of hookworm species in the humans revealed 50.8% of the hookworm positive participants were infected with *A. ceylanicum*, and 51.6% were infected with *N americanus*. Infection with *A. duodenale* was rarely found (3.2%).

Strikingly, almost all (94.4%) of the infections were by a single species. A co-infection by the two most detected species, *A. ceylanicum* and *N. americanus* was only observed in four persons. One possible explanation for these high rates of single infections with *A. ceylanicum* might be the high prevalence of *A. ceylanicum* in the dogs. It has been shown that close contact with infected dogs was shown to be significantly associated with human infection with *A. ceylanicum* (Ngui et al. 2012). Furthermore, these findings also support the hypothesis by Traub and colleagues (Traub 2013) that anthroponotic hookworms may have a cross-protective role in expelling and preventing the subsequent establishment of *A. ceylanicum* hookworms via a Th2 response (Croese and Speare 2006). The infection itself regulates the major immunologic acting against incoming L_3 and L_4 hookworm infection (Behnke et al. 1997). The presence of a stable and long-lived (3–6 years) infection with anthroponotic species may play a role in providing an unsuitable environment for the establishment of incoming larvae of another closely related (albeit suboptimally host adapted) species — in this case, *A. ceylanicum*. If the burden of anthroponotic hookworm species is reduced, this may also have the added advantage of easing density dependent intraspecific competition for limited resources within the intestinal niche (Paterson and Viney 2002), leading to an opportunistic establishment of *A. ceylanicum*.

The analysis of the cox-1 gene of *A. ceylanicum* showed that *A. ceylanicum* isolated from humans and dogs form two genetically distinct groups, which in line with previous findings (Ngui et al. 2013). There are isolates specific to humans and isolates specific for dogs and cats. In our study, we demonstrated that most *A. ceylanicum* isolated from humans clustered

within the zoonotic haplotype. This indicates that there is a cross-transmission from dogs to humans is occurring (Inpankaew T; Schär F 2014).

For the control of transmission, it is important to assess infection status of the animals as well. As a parasite host and reservoir, they are a potential source for transmission to humans. This is especially true for the domestic animals, which play an important part in the everyday life of inhabitants in rural villages. Often, the inhabitants live closely together with their animals. Animals can harbour parasites that have zoonotic potential and can form patent infections in humans as well. This zoonotic potential has been documented for several parasite species, including helminthic infections such as *Ancylostoma ceylanicum* (Areekul 1979), *Ascaris* spp. (Zhou et al. 2012), *Trichuris* spp. (Nissen et al. 2012), *Fasciolopsis buski* (Graczyk et al. 2001) and *Echinostoma* spp. (Lan-Anh et al. 2009) as well as protozoans like *Cryptosporidium* spp. (Thompson and Smith 2011) and *Giardia duodenalis* (Traub et al. 2004a).

12.3 Specific aspects of *S. stercoralis:* Improving diagnosis and uncovering transmission dynamics

In this last section of the discussion we focus on aspects of *S. stercoralis* on the individual level. We have conducted three studies uncovering specific details about the diagnosis of strongyloidiasis and about the dynamics of transmission.

12.3.1 Improving diagnosis – the excretion pattern of *S. stercoralis* larvae

While for other intestinal helminths detailed egg excretion pattern studies were performed, e.g. for liver flukes (Lovis et al. 2012), this information is not available for *S. stercoralis*. Existing patterns can have implications for diagnosis. In our study we assessed for the first time the larval excretion in infected individuals in an endemic setting. The achieve this, we have enrolled 65 persons, of which 39 were *S. stercoralis* positive. The other 26 individuals were from the same households as the infected individuals and acted as control. We obtained a stool sample on seven consecutive days for all persons enrolled. We have modified the Baermann method to estimate the larvae per gram (LPG) stool that are excreted.

We tried to detect a possible pattern in the excretion of larvae with the help of a local maxima estimation. Yet, we could not detect a pattern in the fluctuation of different LPG-counts, which would indicate that the amount of larvae excreted is random. This could be because seven days might be a too short time-sequence, or because of the comparably small sample size, or because there is in no pattern as such, and excretion of larvae is in fact random. We

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could further demonstrate that persons with high-intensity infection (>10 LPG) excreted larvae every day. They therefore pose no problem for diagnosis, as an infection could be rightly diagnosed each day. In individuals having low-intensity infections, we could demonstrate that excretion of larvae can be absent for up to two consecutive days. In surveys applying single day stool examinations, these individuals would wrongly be diagnosed as infection free. Additionally, one person from the *S. stercoralis*-negative control group was found to be infected on the third day of examination. These contributions further emphasize the need to sample more than one sample per person to increase sensitivity and to avoid false-negative results. It is favourable to analyse the samples on consecutive days, as we could not establish a pattern of excretion that would indicate a regular periodicity in which the larvae are excreted (Schär et al. 2014b).

At the individual level, the major challenge for diagnosis, as demonstrated, are the low-intensity asymptomatic/uncomplicated cases of strongyloidiasis. In endemic regions, they can constitute up to 50% (Concha et al. 2005), whereas high–intensity infections have been demonstrated to have a higher morbidity (Khieu et al. 2013b). The use of multiple sampling and the increase in sensitivity has been documented (Khieu et al. 2013a; Knopp et al. 2008; Liu and Weller 1993; Marti and Koella 1993; Nielsen and Mojon 1987; Sato et al. 1995b; Schär et al. 2014b; Uparanukraw et al. 1999; van der Feltz et al. 1999). Our results could confirm these findings, and add for the first time information about the dynamics of larvae excreted by individuals infected with *S. stercoralis* in an endemic field setting.

12.3.2 Improving diagnosis – New molecular diagnostic methods

Besides the classic coprological methods, newly developed molecular diagnostic methods could constitute an alternative for detection of *S. stercoralis* in stool samples. The mainstay for the molecular analysis is detection with the help of the polymerase chain reaction (PCR).

We have therefore evaluated a PCR assay for the detection of *S. stercoralis*. From our school-based study in Kandal (Khieu et al. 2013a), we randomly selected a subset of 218 samples. We have performed a real-time monoplex PCR-assay, based on the PCR developed by Verweij and colleagues (Verweij et al. 2007; Verweij et al. 2009). We compared the performance of the PCR with our results obtaining with the BM and KAP method. PCR showed the highest sensitivity when compared to samples that were positive by both coprological methods (88.9%). The overall sensitivity of the PCR was 61.0% (Schär et al. 2013a).

The PCR replicates specific parts of DNA or RNA sequences. Specific primers are used to specify the target for multiplication. Different PCRs and primers have been described for the detection of *S. stercoralis* (Kramme et al. 2011; Moghaddassani et al. 2011; Repetto et al.

2013; Sitta et al. 2014; Sultana et al. 2013; Verweij et al. 2007; Verweij et al. 2009). The PCR for detection of *S. stercoralis* has proofed its principle, and showed a high sensitivity. Yet, all these studies evaluated the PCR on proven cases of strongyloidiasis. Therefore, information about the sensitivity and applicability of PCR in asymptomatic, unconfirmed cases of *S. stercoralis* is lacking. Accordingly, in our study, we compared the PCR with the two gold standard coprological methods, the KAP and BM, in detecting unproven cases of *S. stercoralis*. We established a sensitivity of 61.0% for the PCR. Recently, Knopp and colleagues also compared the performance of PCR compared with only the BM for the detection of *S. stercoralis*. They reported sensitivities ranging from 12 – 31% (Knopp et al. 2014). This compared to our study, which reported a sensitivity of 88% when comparing the PCR and BM only. One possible explanation might be, that the infection intensities of positive individuals was considerably higher in our study compared to the individuals diagnosed in the study of Knopp (Knopp et al. 2014). The lower intensities might decrease the amount of DNA present and therefore making it more difficult to extract DNA and subsequently for the PCR to detect infection, increasing the number of false-negative results. Compared to the BM, with the PCR a much smaller amount of stool is used for analysis. Recently, it was documented that the DNA extraction process can be optimized. Repetto and colleagues improved the yield and purity of extracted DNA by including a step of incubation with glycine-SDS buffer and mechanical disruption. They further added a bovine serum albumin for neutralization of inhibitors present in the stool during PCR (Repetto et al. 2013).

The PCR is still relatively new for diagnosis of *S. stercoralis*. Major constraints are the comparably high costs and the sophisticated material needed for the performance of PCR. This holds especially true in resource poor settings or if large-scale examinations are to be undertaken. Our PCR was performed in the laboratories at Swiss TPH in Basel. For individual diagnosis, the PCR can be an adequate alternative. With the development of multiplex-PCR assays that can screen for several parasites at the same time, the cost-effectiveness can be increased (Verweij et al. 2007). Other PCR assays, such as the LAMP (loop-mediated isothermal amplification) PCR have been developed for helminth infections, i.e. for *Opisthorchis viverrini* (Le et al. 2012) and might provide a future alternative for diagnosis. Additionally, there are the first surveys evaluating coproantigen assays for *S. stercoralis* (Sykes and McCarthy 2011).

12.3.3 *S. stercoralis* and transmission dynamics

To assess transmission dynamics, molecular analysis of parasites is an indispensable tool. We therefore aimed at analyzing the genetic structure of *S. stercoralis* populations within a particular location. We sequenced 269 individual worms collected from 29 individuals. We could identify three different genotypes of *Strongyloides* in our study population, co-existing

sympatrically within the same host. Yet, we were not able to detect heterozygous worms, which would indicate that there is no interbreeding (Schär et al. 2014a).

A possible explanation would be that sexual reproduction is rare or absent in at least certain lines of *S. stercoralis* in our study area. This could be either because successful reproduction occurs exclusively through the parthenogenetic direct cycle or because reproduction in the free-living generation is non-sexual, in spite of the existence of males. Indeed, based on cytological studies, it was proposed that reproduction in the free-living generations of multiple species of *Strongyloides* is by sperm dependent parthenogenesis (pseudogamy) (for *S. ratti* (Nigon and Roman 1952); for *S. papillosus* (Triantaphyllou and Moncol 1977); for *S. stercoralis* (Hammond and Robinson 1994)). By applying molecular genetic approaches, this hypothesis was disproved and sexual reproduction has been demonstrated for *S. ratti* (Viney et al. 1993) and for *S. papillosus* (Eberhardt et al. 2007). So far, no genetic investigation has been conducted with *S. stercoralis* and pseudogamic reproduction remains a possibility in this species.

In the case of *S. stercoralis* and other nematodes, the small ribosomal sub-unit (SSU) rDNA, a highly conserved sequence is used for phylogenetic studies among and within species (Blaxter et al. 1998; Floyd et al. 2002; Hasegawa et al. 2009; Herrmann et al. 2006). For the genus *Strongyloides,* SSU sequences have been used for molecular taxonomy (Dorris et al. 2002; Eberhardt et al. 2008; Hasegawa et al. 2009; Hasegawa et al. 2010). Only a few studies on within-species variations in the SSU and in mitochondrial DNA sequences have been conducted on *S. stercoralis,* originating from different locations and hosts (Hasegawa et al. 2009; Hasegawa et al. 2010; Hu et al. 2003; Koosha et al. 2009; Pakdee et al. 2012).

To assess the zoonotic potential of *S. stercoralis* and possible transmission between animals and humans, we conducted an additional study, including 339 worms from humans, 119 from dogs and 68 from pigs. The worms from the animals were collected in the households, were the human worms were collected. First preliminary results show that *S. stercoralis* from humans forms at least three separate populations. In dogs, *S. stercoralis* forms at least five separate populations. All worms isolated from humans have the same sequence at the hyper-variable region 4 (HVR4), only 9.6% of dogs have the same sequence as in humans. Worms with the same HVR4 genotypes are shared between humans and dogs. All 18S rDNA HVR1 and HVR4 sequences of worms isolated from pigs were different from worms isolated from humans and dogs. These sequence result indicate that *S. stercoralis* in humans and dogs form different populations, as 90% of the dogs have a different nucleotide arrangement at HVR4. Yet, while in humans, the HVR4 sequence most commonly detected in dogs could not be found, the remaining 10% of dogs have the same HVR4 sequence as in

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humans. This may suggest that the human and canine strains of *S. stercoralis* were diverged since the time of domestication and adopted to respective hosts. Yet, there is a chance *S. stercoralis* could be transmitted from dogs to humans and vice-versa, because 10% of the worms have the same sequence in dogs and humans (unpublished, personal communication T. Gudeta and A. Streit).

12.4 Conclusions

With the work presented in this thesis, the following conclusions and observations can be reported:

1. We could demonstrate that the prevalence of *S. stercoralis* is underreported. Since the last review on *S. stercoralis* prevalence, many surveys have been undertaken and new information has been collected. Yet, we showed that there are still many gaps and "black spots" on the world map remaining today. Furthermore is the available information very heterogeneous, which makes it challenging to summarize it.

2. Diagnostic methods most commonly applied in studies on STH infections today are not appropriate for the detection of *S. stercoralis*, which is one of the main factors for the underreporting of the prevalence.

3. We conclude that the high sensitivity coprological methods should be integrated in the routine diagnosis in helminth control programs. Especially the Baermann method offers a comparably good applicability in field settings, with the only contraints being the big amount of stool that has to be handled and the need for running water.

4. Detailed studies focusing on risk factors like HIV-, and HTLV-1 infection are scarce, and the associations between *S. stercoralis* and those infections are not entirely clear.

5. Ivermectin is the best available treatment. Our studies further confirmed the effectiveness and safety in application of ivermectin. As ivermectin already has been applied in mass drug administration (MDA) programmes, for instance in the treatment of lymphatic filariasis, inclusion of ivermectin in helminth control MDA-programmes is an option to be considered in endemic areas.

6. We conclude that alternative treatment options, such as Tribendimidine, which showed good anthelminthic effects, need to be further evaluated for the impact on *S. stercoralis* infection.

7. With our large-scale studies conducted in Cambodia, we could demonstrate the presence of high infection rates of *S. stercoralis*, never been reported at that high level in Cambodia. It shows that applying good sensitivity diagnostic methods on consecutive days gives prevalence rates that are a closer to the true prevalence and

considerably higher than what has been previously reported. We conclude that this is true for most *S. stercoralis* endemic settings and hence, the true prevalence is likely to be higher than the numbers reported in studies with low sensitivity diagnostic methods.

8. We demonstrated the high prevalence of intestinal multiparasitism in rural villages in Cambodia, not only in humans but also in domestic animals.

9. We documented that infection with *A. ceylanicum* is rampant in humans in rural villages, and we showed the potential for zoonotic transmission between dogs and humans. For prevention and control, integrated approaches are needed, including not only the humans but the animals as well.

10. We could demonstrate and there is no pattern in the excretion of *S. stercoralis* larvae and that low-intensity infections can easily be missed if only single stool samples are analysed, as the individuals can have excretion-free days. This further emphasizes the importance of analysing samples of the same individual on consecutive days to increase sensitivity of the diagnostic methods and to subsequently better estimate the true prevalence

11. We tested and evaluated a novel molecular diagnostic technique (PCR) and conclude that it is a suitable option for individual diagnosis, but is difficult to apply in field settings.

12. We could demonstrate three different genotypes of *Strongyloides* in humans in Cambodia.

12.5 Next steps of research

Our work and the obtained results call for the following research needs:

Section 1:

1. *S. stercoralis* remains underreported and the missing data on prevalence calls for studies to create further evidence of the real importance of this parasite.

2. There is a need for large-scale community-based surveys to focus on *S. stercoralis*, applying the best available diagnostic methods. This is especially true in most endemic settings.

3. Integration of routine diagnosis with the appropriate diagnostic methods available in control programs for helminthic infections in endemic settings is warranted.

4. Ivermectin was shown to be the best available drug, and future studies should evaluate the impact of inclusion of Ivermectin in nation-wide MDA-programs on helminths.

5. The high costs of Ivermectin are a factor that hinders the widespread use of this drug for treatment of *S. stercoralis*. Studies on the efficacy and applicability of alternative drugs are needed. There is the need for follow-up on the most prominent candidates for alternative treatment options, such as Tribendimidine and Mirazid.

6. We could demonstrate associations between certain risk factors and infection with S. stercoralis. Detailed studies that shed further light into these associations between strongyloidiasis and associated risk factors such as HIV and HTLV-1 are needed. This includes studies on population level as well as studies on the individual level.

7. Control of infection at household level should not only focus on human disease, but include the domestic animals as well.

Section two:

1. We could further demonstrate the importance and prevalence of multiparasitism in *S. stercoralis* endemic settings, in humans as well as in animals. The zoonotic potential of *S. sercoralis* and *A. ceylanicum* have been analysed, yet there is a further need for quantitative analysis of this zoonotic transmissions in comparison to the anthroponotic transmission

2. For the quantitave assessment of zoonotic transmission, further molecular analysis is essential to uncover specific details of transmssission dynamics.

3. For the control, it is important to further determine which animals have the highest potential for sustaining the zoonotic transmission cycle. They need to be specifically targeted and included in control programs, i.e. a One Health approach for control is warranted.

Section three:

1. Our study on the excretion showed the challenges for diagnosis of low-intensity infections. Further detailed analyses are needed, also on how acute morbidity is associated with low- and high-intensity infections.

2. Indepth analysis on excretion of larvae should also influence evaluation of diagnostic methods, i.e. like ERR (egg reduction rate) in other helminths. Accordingly, a larvae reduction rate (LRR) for *S. stercoralis* can be used to assess treatment effect and diagnostic sensitivity.

3. We demonstrated the applicability of PCR as a diagnostic tool, yet it is not ideal, especially for the use in large-scale surveys in endemic settings. Studies for

evaluating novel approaches such as multiplex-PCR or LAMP-PCR are needed as they might provide a more cost-effective option with a better applicability in field settings.

4. Other new diagnostic possibilities like coproantigen tests for *S. stercoralis* have been developed and might facilitate diagnosis. Additionally, development of rapid tests for the detection of *S. stercoralis* that would show a high sensitivity, easy applicability and good cost-effectivenss would be ideal.

5. Further studies on the genetics of *S. stercoralis* are needed, also assessing the possible genetic exchange occuring in the free-living cycle of *S. stercoralis*.

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