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Prevalence of *Strongyloides stercoralis* infection in adult HIV–positive patients and comparison of specificity and sensitivity of five different methods to detect a current infection in Mwanza Province /Northern Tanzania

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A study to assess the prevalence of *Strongyloides stercoralis* infections among adult HIV–positive patients by comparing sensitivity and specificity levels resulting from Agar plate culture, the Harada Mori, FPC, PCR and ELISA techniques applied in the Bugando referral hospital in Mwanza Province, Northern Tanzania.

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Die Promovendin ist Ärztin.

Gewidmet ist diese Arbeit meinen Eltern

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I. Operational Definitions

IRIS - Immune Reconstitution Inflammatory Syndrome

According to the consensus criteria IRIS is characterized by a clinical deterioration of an HIV-infected individual who recently started antiretroviral treatment. [1] The deterioration cannot be explained by a previously recognized infection, side effects of the medication, treatment failure or non-adherence. It is usually accompanied by an increase in CD4 cell count and rapid decrease of the viral load.

Strongyloides Hyperinfection syndrome

Hyperinfection syndrome represents an accelerated auto infective cycle with large numbers of rhabditiform larvae transforming into infective filariform larvae within the human gastrointestinal tract. [2] It is usually due to pronounced and protracted immunosuppression. There can be a high number of migrating larvae, but they do not affect organs outside the natural life cycle.

Disseminated Strongyloidiasis

In disseminated Strongyloidiasis the auto infective cycle is grossly accelerated with larvae migrating into various organs and tissues, not restricted to the natural route of migration. [2] Organs like liver, kidneys, heart and brain can be affected. Disseminated Strongyloidiasis has a high case fatality rate if not recognized and treated adequately.

II. Abbreviations

ART   Antiretroviral treatment

ASL   Automated Lab Solution

BMC   Bugando Medical Centre

CDC   Center for Disease Control and Prevention

CTC   Care and Treatment Centre

CUHAS   Catholic University of Health and Allied Sciences

DNA   Desoxyribonucleic acid

ELISA   Enzyme-linked Immunosorbent Assay

FPC   Faecal parasite concentrator (Evergreen Scientific)

HIV   Human Immunodeficiency Virus

HTVL 1   Human T Lymphotropic Virus 1

IgE   Immunoglobulin E

IRIS   Immune Reconstitution Inflammatory Syndrome

NIMR   National Institute of Medical Research

OR   Odds Ratio

PCR   Polymerase Chain Reaction

PASW   Predictive Analysis Soft Ware

WHO   World Health Organization

III. Abstract

a   Background

S. stercoralis is a helminthic parasite which is common in tropical and subtropical regions. It causes a persistent but often inapparent infection in humans. In the state of a protracted immunosuppression this parasite can cause a life-threatening hyperinfection syndrome. Most often the hyperinfection syndrome was found after prolonged high dose corticosteroid treatment.   In HIV-infected individuals high dose corticosteroids are used for the treatment of the immune reconstitution inflammatory syndrome (IRIS) or as adjunct treatment in the treatment of meningeal or pericardial tuberculosis. Case reports from Tanzania demonstrate that Strongyloidiasis is prevalent not only in coastal regions but also in the Lake province of Tanzania. However, data on the local prevalence of *S. stercoralis* infection based on sensitive techniques are scanty, especially in HIV-infected individuals.

b   Objectives

The main objective of this study is to provide data on the prevalence of *S. stercoralis* infections in the adult HIV-infected population attending the Bugando Medical Centre for medical care.

Specific objectives of the study are the comparison of the sensitivities and specificities of five different methods in detecting *S. stercoralis*. Four methods to detect *S. stercoralis* larvae used stool samples; one method to detect *S. stercoralis* antibodies required blood samples.

The results of the prevalence study have been related to an individual in depth questionnaire in order to verify the results to standard risk factors as described by *Echazu et al.* and *Khieu et al.* [3, 4].

c   Methods

The study used the Agar-plate-culture-technique and a modified Harada-Mori-culture-technique for the direct detection of helminthic larvae in the

collected faecal samples. In addition, a recently described PCR-assay from faecal specimens and an ELISA for *S. stercoralis* antibodies have been applied. The Faecal Parasite Concentrator (FPC) stool concentration technique was used for the differential diagnosis of other intestinal helminthic parasites.

d   Implications

The results of the study may influence the current treatment guidelines for HIV-infected patients in case that a relevant prevalence of *S. stercoralis* infection is found. Then, prior to a prolonged iatrogenic immunosuppression -like the high dose corticosteroid treatment for IRIS- a prophylactic anthelminthic treatment capable to eradicate a *S. stercoralis* infection could be recommendable.

e   Results

The prevalence of a current *S. stercoralis* infection using the PCR as a gold standard was 5.4%. The Agar plate method showed positive results in 19 out of 278 cases (6.1%), the modified Harada Mori technique in 13 of 278 (4.7%) cases. With PCR as gold standard the sensitivity of the agar plate method was 60%, the positive predictive value 47.4%, the specificity 96.2% and the negative predictive value 97.7 %. The sensitivity of the Harada Mori technique was 36.4%, the positive predictive value 30.7% with a specificity of 96.4% and negative predictive value 97.1%. The modified Harada Mori technique allowed in principal the morphological identification of nematode larvae. Microscopic analysis showed a specificity of 100% and a sensitivity of 46.7%. Antibodies were detected in 45 of 278 cases 16.2% by ELISA, with a sensitivity of 92.9% and a specificity of 87.8%.

1. Introduction

According to the UN AIDS gap report 2016 about 1,4 million people of Tanzania are infected with HIV resulting in a total HIV prevalence of 5,4 % among adults.[5]

A study in Tanzania from *Msisha et al.* estimates the prevalence rate in a range from 4.8% to 8.1% and shows that the region around Mwanza has an, above average HIV prevalence in Tanzania as shown in figure 1 on page two. The WHO reports that the Republic of Tanzania was among the 22 countries in the world in 2014 with the highest prevalence of tuberculosis. Furthermore, it showed that out of the 63151 reported cases of Tuberculosis 35% have been HIV positive.[6]

The national guideline of the AIDS Control Program initiated by the government of Tanzania consists of a HIV therapy and treatment program which aims to enrol all HIV infected Tanzanians. According to the UNAIDS Gap report 2016 in year 2015 about 53% of HIV infected Tanzanians adults had access to Anti-Retroviral Treatment (ART). This remarkable achievement and process is still enhanced by the government authorities in order to reach out to all HIV infected inhabitants. [5]

Figure 1: HIV prevalence in the United Republic of Tanzania

Source: *https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2789284/figure/F1/*
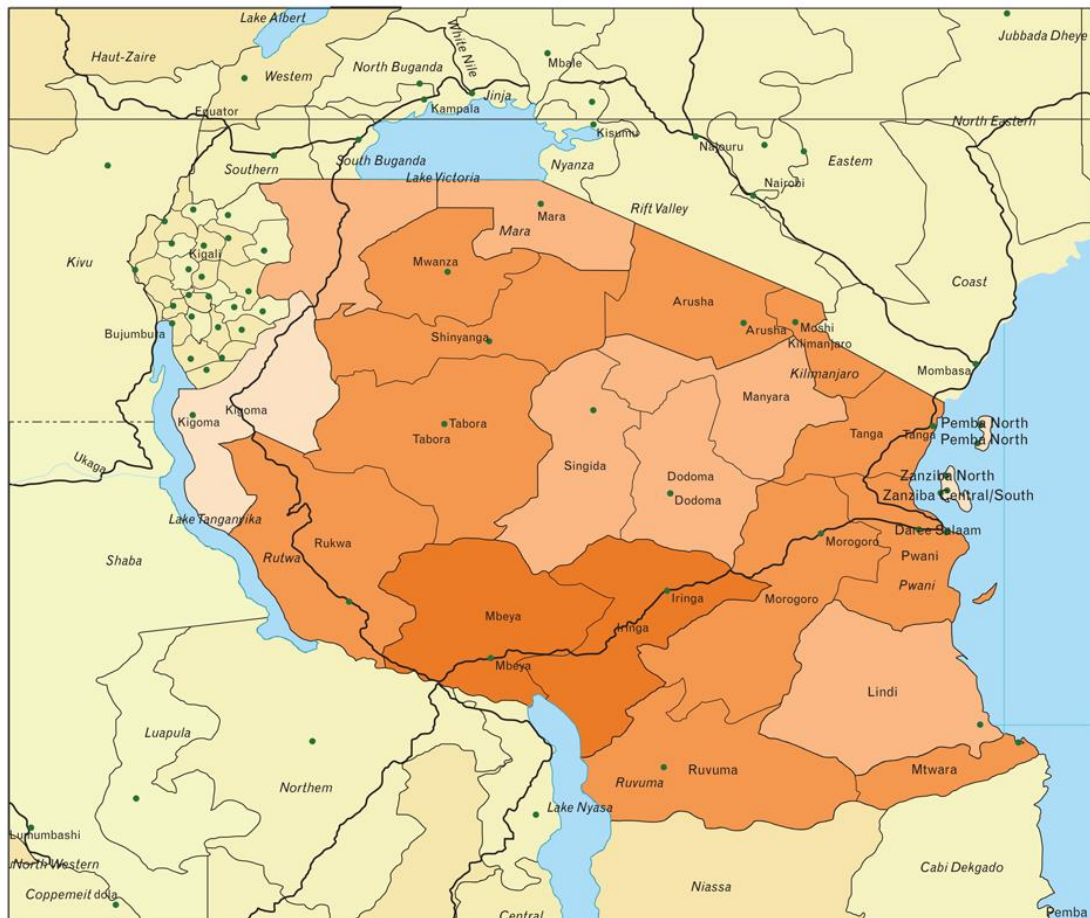
Regional HIV prevalence (in %): ▭ 0.0 – 1.6; ▭ 1.6 – 4.8; ▭ 4.8 – 8.1; ▭ 8.1–15.5

However, since the beginning of ART, increasing numbers of patients who developed an immune reconstitution inflammatory syndrome have been reported. [7-9] *French et al.* reported in his study the possibility of developing an IRIS after receiving ART, which is particularly high in patients who are having a Tuberculosis co- infection.[8].

Asdamongkol reported that *S. stercoralis* infection may lead to a potentially fatal hyperinfection syndrome.[10]

2. Problem statement and objectives of the study

2.1 Problem statement

Strongyloidiasis is a commonly underdiagnosed and a highly neglected parasitic infection due to its difficult diagnosis. It may become deadly for HIV-infected patients who receive ART and high dose corticosteroid treatment. This corticosteroid treatment of HIV patients is necessary for the treatment of an immune reconstitution inflammatory syndrome (IRIS). E.g. in a study performed in South Africa a cumulative incidence of IRIS of 22.9% is described within HIV positive patients starting ART. [11] On the webpage of the AIDS Institute of the New York State Department of Health the experts recommend the application of prednisone with a dosage of 1-2 mg / kg of body weight administered daily during a period of 1-2 weeks and tapered off thereafter over weeks.[12] This application can lead to the *S. stercoralis* hyperinfection syndrome in case of a previously unrecognised oligosymptomatic carrier state. This dangerous situation is often overlooked since the symptoms of severe IRIS and those of the *S. stercoralis* hyperinfection syndrome are overlapping widely. Therefore, *Mejia and Nutman* recommend that *S. stercoralis* treatment should be considered for all cases of corticosteroid treatment for IRIS. [13]

Medical studies from *Marchi Blatt and Cantos, Mariam et al. and Schar et al.* report that there also appears to be a higher prevalence of *S. stercoralis* among HIV-positive patients. [14-16]

The diagnosis of *S. stercoralis* infection is difficult; it needs effective and well- equipped laboratories, requires expensive materials, the mastering of difficult techniques and is time consuming. Currently, these preconditions are often not available for routine use in the hospital settings in developing countries like Tanzania. Therefore, the prevalence of *S. stercoralis* in HIV- infected patients has not been adequately investigated and is generally underestimated. Furthermore, treatment regimens used

to treat intestinal helminthic infections do not cure *S. stercoralis* infection sufficiently. [17]

2.2 Objectives of the study

Although the rate of HIV infected people in the area around the Lake Victoria is above national average in Tanzania, no data on the prevalence of *S. stercoralis* infection among HIV patients in this area of Tanzania can be found.

Prevalence data on *S. stercoralis* in HIV-infected patients obtained by sensitive methods which could be used as a justification for empirical treatment are lacking for Tanzania. Only recently, an unusual presentation of a *S. stercoralis* infection in a HIV-positive woman, -showing symptoms of an upper gastrointestinal bleeding- at Bugando Medical Centre, Mwanza, has been published by *Jaka et al.* [18]. This report indicates that *S. stercoralis* occurs in the local population of Mwanza Province in Tanzania.

The objectives of this study are to identify the prevalence levels of *S. stercoralis* infection in HIV-infected adults and to assess the method which provides the highest possible yield. This second objective shall be achieved by comparing four different methods to detect *S. stercoralis* larvae from stool samples as well as *S. stercoralis* antibodies from blood samples using an ELISA. The sensitivities and specificities of an ELISA, a modified Harada Mori technique, the FPC- technique, the Agar plate culture and the realtime PCR were compared. Various reports indicate that the PCR has a high sensitivity of nearly 100% in detecting *S. stercoralis* DNA in stool samples. Therefore, realtime PCR was used as the assumed "Gold Standard" to calculate specificity and sensitivity of the other methods. [19-22] The identification of proxy-indicators for an increased likelihood of *S. stercoralis* infections will be assessed through the correlation between the analysed prevalence data with selected factors generated from the socio-economic and risk data of the patients. The

results of this study may contribute to better understand *S. stercoralis* prevalence and infection and its impact on HIV patients as well as to future treatment strategies for advanced HIV-infection.

3. Literature Review

3.1 Epidemiology

S. stercoralis is prevalent mainly in tropical and subtropical environments. On the webpage of the WHO it is stated that about 30-100 million people are infected with this potentially life-threatening parasite.[23]

The inaccuracy of this WHO estimate of the global *S. stercoralis* prevalence is a consequence of the insidious nature of this helminthic infection, unspecific symptoms and its difficult detection by routine techniques.

A study conducted in a village in Yunnan province in China with careful examination of repeated samples from a random population using different diagnostic methods resulted in an overall prevalence of 11.7%. [24] A study by *Yelifari et al.* in northern Ghana showed a prevalence of 10.6%. *Olsen et al.* reported a prevalence of 9-12% in a study from Mozambique using careful examination of direct smears. However they discovered a much higher prevalence of 48% when using a combination of culture techniques and PCR.[25, 26] A recent study of *Knopp et al.* in two villages in Zanzibar / Tanzania detected *S. stercoralis* larvae in 10.3% and in 12.7% of the villagers, respectively, using a combination of both techniques. [27]

In tropical climates a high prevalence of *S. stercoralis* infection has been reported in children. [4] It can be assumed that the infection already often takes place during childhood, mainly under the living conditions in rural tropical areas.

The study from Kenya by *Walson et al.* most likely underreports the true prevalence of *S. stercoralis* infection. As none of the applied laboratory techniques exhibits a reasonable sensitivity. [14],[28],[29]

The study showed a prevalence of 1.3 % of *S. stercoralis* infection using wet mount microscopy, the Kato-Katz-technique and formol-ether concentration technique among HIV-infected patients with a CD4 cell count > 250/µl. [30]

A meta- analysis from *Schar et al.* investigated the prevalence of *S. stercoralis* infection from articles published between January 1989 and October 2011 with information on the prevalence of *S. stercoralis*. The study proves that there is a conspicuous lack of information on the prevalence of *S. stercoralis* in African countries in which environmental, social and socioeconomic conditions are highly predisposing to acquiring a *S. stercoralis* infection. [16, 31]

Figure 2: Prevalence of *S. stercoralis* and intensity of research in tropical countries by number of studies per country

Source: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3708837/figure
/pntd-0002288-g003/
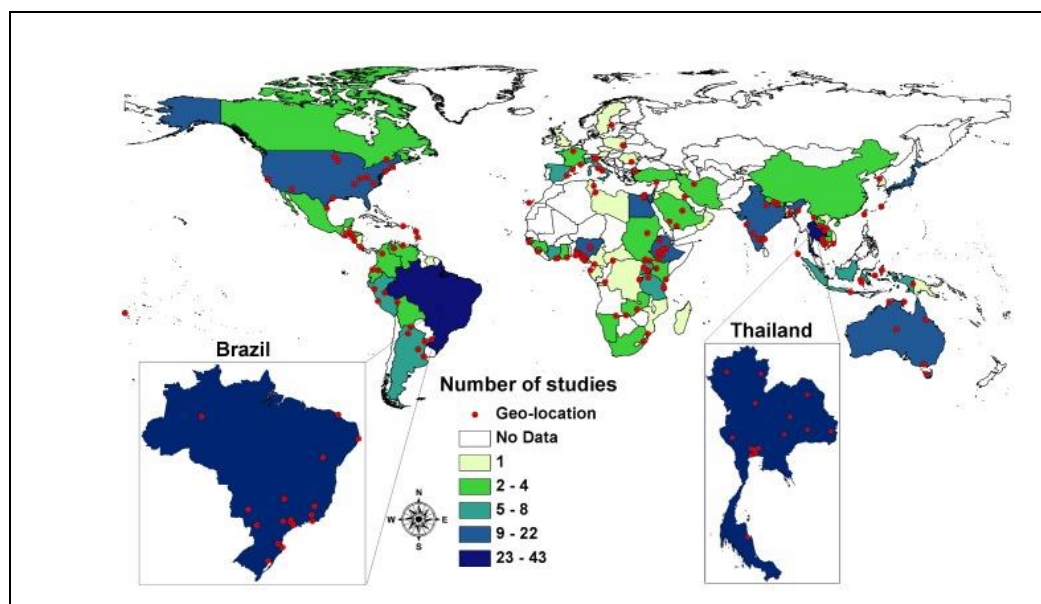
The study by *Schar et al.* indicates that 10% to 40% of the population in many tropical and subtropical countries are afflicted by a *S. stercoralis* infection. Their analysis showed that in most of the investigated studies diagnostic techniques with a low sensitivity were used. Hence, they claim that the prevalence of infection with *S. stercoralis* might be widely underestimated. [16]

Studies by *Knopp et al.* describe in general that, helminthic infections show a spatial distribution with lower prevalence in urban settings. [32]

The numbers and types of studies undertaken for the global estimation of the prevalence of a *S. stercoralis* infection is diverse and heterogeneous and does not give conclusive results. The two following figures show the results of a community based and health systems-based screening study for the prevalence of *S. stercoralis* infections. Figure 3 shows the global results of community screening studies indicating highest prevalence in the tropical countries. Whereas Figure 4 shows the results of studies based on health services data which only focused on patients with symptoms. Figure 4 confirms the expected high prevalence in the developing countries in the tropics but also indicates unexpected high prevalence in developed countries such as France and USA. [25]

3.2   The Strongyloides genus

The *Strongyloides* genus contains about 50 different species infecting the gastrointestinal tract of vertebrates, e.g. mammals, birds, reptiles and amphibians. It can be considered that the specific denomination may have been ascribed mostly by the host species in which the parasite was found.[33]

An infection with *Strongyloides* is relatively common in wild and domestic animals. E.g. *S. ratti* showed a prevalence of 62% in a study that investigated rats in the UK 1998.[33]

Two species of the *Strongyloides* genus can infect humans. *S. stercoralis* and *S. fuelleborni*. Among them *S. stercoralis*, a helminthic soil-transmitted parasite, is the most common and globally distributed pathogen and of clinical importance.

Figure 3: Prevalence of *S. stercoralis* infection by country based on community studies.
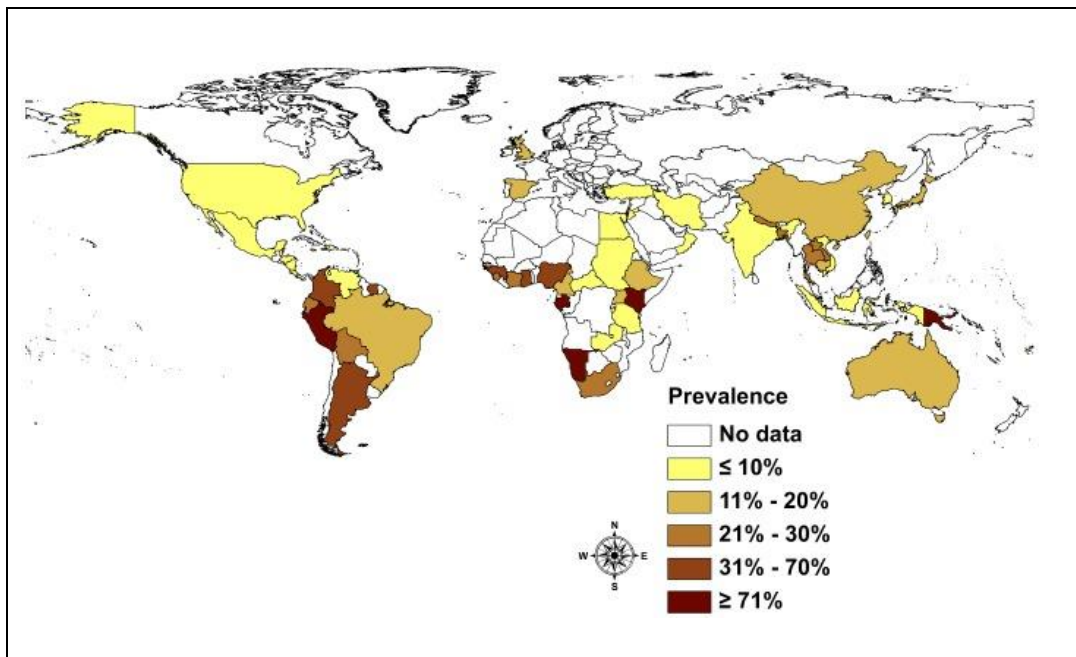
Figure 4: Prevalence of *S. stercoralis* infection by country based on health services studies.
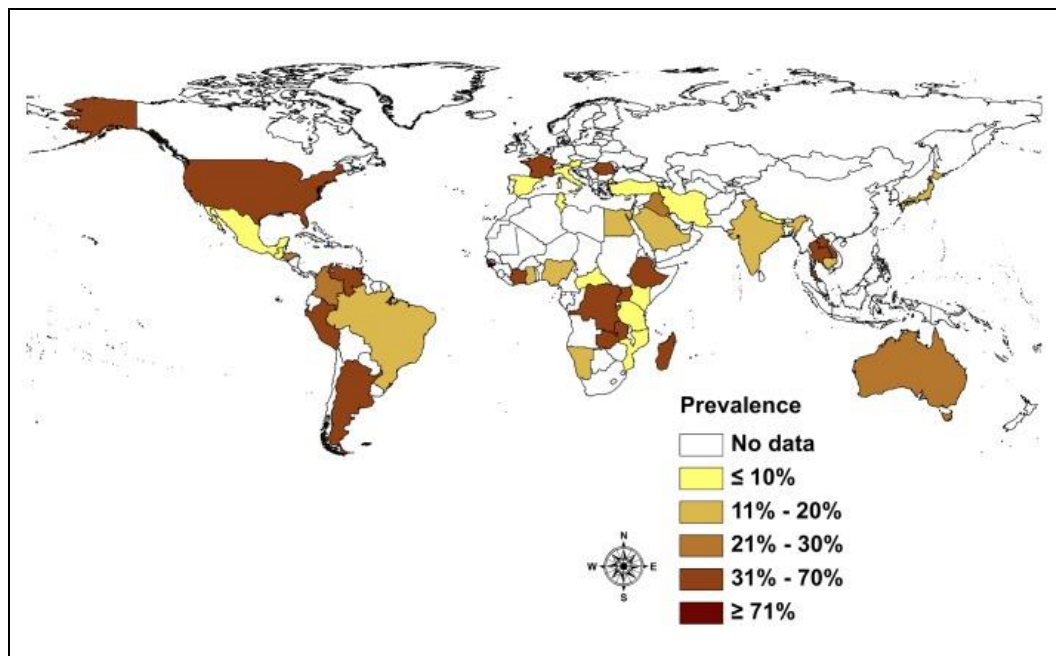
Whereas *S. fuelleborni* infection is mostly found in African primates and can be shared with humans. *S. fuelleborni kellyi* is found exclusively in humans in New Guinea.[33, 34]

According to the WHO an estimated 30 - 100 Million people are suffering from an infection with *S. stercoralis* worldwide, but precise data on the prevalence in the endemic countries are unknown. The highest prevalence of *S. stercoralis* infection is found in tropical and subtropical regions.[23]

3.3   The life cycle of *S. stercoralis*

The life cycle of *S. stercoralis* as shown in Figure 6 is heterogenic with the possibility to alternate between a parasitic cycle as well as a free-living non-parasitic cycle. [35] This is of advantage for the parasite as it allows it's surviving in the soil in the absence of a suitable host. The infection of humans occurs by active penetration of the infective filariform larvae through the intact skin. It is assumed that potential hosts walk barefoot or sit on the soil which enables direct skin contact. The penetration of the skin is facilitated by a potent histolytic protease secreted by the infective larvae. Via the lymphatic system and venules, the larvae reach the pulmonary circulation, then penetrate into the alveolar spaces and migrate up the bronchial tree. After reaching the pharynx they are swallowed and finally reach the small intestine. Embedded in the mucosa they mature to female adults. The females measure about 2-2.5 mm in length x 0.034 mm in width. [35] They produce up to 2000 eggs per day by parthenogenesis. The eggs already contain larvae which hatch during their further intestinal passage. The common diagnostic stage in faecal samples is therefore the rhabditiform L1 larva. According to *Piekarski* the shortest reported prepatent period is 17 days.[36] Still within the intestine the rhabditifom L1 larvae can transform into infective filariform L3- larvae and penetrate the intestinal mucosa or perianal skin. This autoinfection is responsible for a persistent, life-long infection if the individual is not treated appropriately.

Apart from *S. stercoralis* autoinfection in human parasitic nematodes is only known from *Capillaria philippinensis* infections. [37]

The autoinfection cycle is a thread especially to severely immunosuppressed individuals caused by HTLV-1 infections, antineoplastic chemotherapy or prolonged high dose corticosteroid treatment. Rhabditiform larvae which are shed with the faeces may develop into free living male and female adults that mate; the sexual reproduction is only found in the free-living cycle in the soil. Depending on the temperature larvae hatching from the eggs develop within 7-10 days into infective L3- larvae, ready to enter the parasitic cycle again. They remain viable in the moist soil for many weeks. The free-living adults die after one generation and do not persist in the soil. The following figure 5 shows the lifecycle of *S. stercoralis.*

Figure 5: The lifecycle of *S. stercoralis*

Source: https://www.cdc.gov/parasites/strongyloides/biology.html
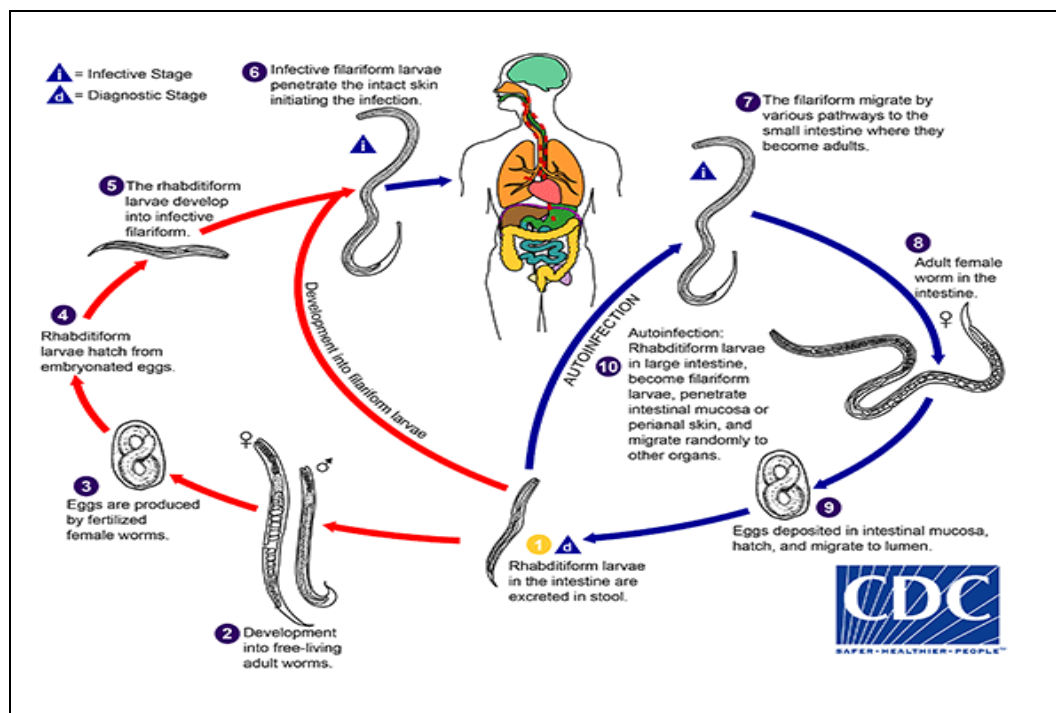
3.4 The clinical features of *S. stercoralis*

The clinical features of Strongyloidiasis as described by *Bannon et al.* are very variable ranging from asymptomatic carrier state over oligosymptomatic disease to hyperinfection syndrome and disseminated disease with high mortality. [38]

Nutman et al. reported that common gastrointestinal symptoms are abdominal pain and diarrhoea.[39] It was found by *Atul et al.* that the local inflammatory reaction of the intestinal mucosa may induce a malabsorption syndrome. [40] Pulmonary symptoms may occur during lung passage of filariform larvae and present as pneumonitis associated with peripheral blood eosinophilia also known as Loeffler's syndrome. Dermatological manifestations may occur as petechial hemorrhages at the port of entry of infective larvae, accompanied by intense pruritus, congestion, and edema. A larva currens syndrome and urticarial rashes are common in disseminated disease. [41, 42]

Studies by *Nutman et al.* describe that disseminated Strongyloidiasis is the result of immunosuppression which is most often of iatrogenic nature. It can present as an acute shock-like illness with gram-negative septicaemia due to bacterial translocation by the migrating parasites.[39] *Fardet et al.* reported that in a case series of 151 patients with disseminated Strongyloidiasis due to corticosteroid treatment, 91% of patients presented with non-specific gastro-intestinal symptoms, 54% showed low grade fever, and 39% had developed a severe bacterial or fungal infection. [43]

Secondary bacterial and fungal infections are common due to a leakage of gut flora from a bowel damaged by moving rhabditiform larvae and invasive L3- larvae carrying enteric bacteria on their outer surfaces. This could lead to an immune activation within HIV-positive patients and therefore to a progression of AIDS-defining symptoms. [44]

3.5 Treatment of Strongyloidiasis

A study performed in Zanzibar investigated within 301 schoolchildren the effectiveness of 400 mg albendazole for three days to cure a *S. stercoralis* infection and compared this drug to the effectiveness of a single dose ivermectin of 200 microgram/ kg. The results showed that only 45% of the infections could be cured after the application of albendazole whereas 89% of the infections could be cured with ivermectin.[17].

To achieve a cure, prolonged and repeated courses of anthelminthic agents need to be administered.

Gann et al. and *Marti et al.* describe the effectiveness of one single dose ivermectin to eradicate the infection.[17, 45] Thus, it must be considered that these results were achieved by often using insensitive methods to visualise the absence of larvae. Recent studies by *Page et al.* show the need to implement an ELISA in which a seroconversion should occur. [46] Therefore, newer publications by WHO recommend therapy with ivermectin on two successive days compared to a previously recommended single dose. [47]

Currently, the treatment of choice is ivermectin 200 µg / kg on 2 following days. Treatment with albendazole 400 mg bid for 7 days is an alternative, but inferior choice. The required dosage of albendazole is by far higher as the commonly applied dosage used in mass treatment campaigns for common soil transmitted helminthiases like *Ascariasis, Trichuriasis or* Hookworm disease but still inferior to ivermectin. Therefore, it can be assumed that mass treatment with albendazole has only a very limited impact on the prevalence of Strongyloidiasis.

Moreover albendazole targets mainly the adult parasites. [39]

According to various studies treatment with ivermectin is superior to albendazole for the cure of *S. stercoralis* infection. The national guidelines of Tanzania from 2007 recommend for the therapy of an infection the use of thiabendazole 25mg/ kg body weight twice daily for three consecutive

days. [48] However, thiabendazole is hardly available for human use in most of the countries nowadays.

Therefore, the new guidelines in Tanzania from May 2013 recommend the use of ivermectin 200µg/ kg body weight for two days. [48]

Studies describe that the cure effectiveness of thiabendazole is similar to the effectiveness of ivermectin. But under therapy with thiabendazole a higher occurrence of adverse events such as disorientation, fatigue and nausea has been observed.[45, 49] Ivermectin is the treatment of choice in disseminated Strongyloidiasis and the hyperinfection syndrome for a duration of two weeks minimum but should continue until negative stool examinations persist for 2 weeks. [2] [39] Furthermore, a reduction of immunosuppressive therapy should be considered if the hyperinfection syndrome becomes a potential medical emergency. [39]

3.6   Diagnosis of Strongyloidiasis

A comprehensive study was conducted by *Marchi Blatt and Cantos* (2003) in Brazil on the evaluation of different techniques for the diagnosis of *S. stercoralis.* They compared the sensitivities and specificities of five different techniques, the Lutz-, the Formalinethyl- acetate-, the Baermann- , the Harada Mori - technique and the Agar plate culture. In this study the PCR method was not considered. The findings show that the Agar plate culture method is superior in detecting *S. stercoralis* larvae in stool samples. [14] Furthermore, the results indicate that there is a predominance of *S. stercoralis* infection within HIV positive patients compared to HIV negative patients. Other studies done on the efficiency of methods in detecting *S. stercoralis* in stool samples show that the PCR method has a high sensitivity.[22] *Arndt et al* applied the PCR method to detect helminth infections including *S. stercoralis* within HIV positive patients in Kenya. They could prove a much higher sensitivity of the PCR method compared to common microscopy methods. Therefore, these

results indicate that the used PCR method is a highly sensitive tool for diagnosing *S. stercoralis* infection among HIV positive patients.[19]

3.7   Diagnostic tests for *S. stercoralis* infection

Concentration techniques like the SAF-concentration method (Sodium acetate-acetic acid-formalin fixative) are widely used for the demonstration of parasite ova and protozoan cysts. Occasionally, *S. stercoralis* larvae can be detected, but the sensitivity for this purpose is low, depending on the amount of larvae per g of stool.[50, 51]. Therefore, faecal culture techniques are mainly used which provide a higher yield in detecting *S. stercoralis* larvae. The traditional Baermann test, the Harada-Mori culture and the Agar plate culture are the most commonly applied culture tests. All of them are focussing on the detection of helminth larvae, namely Hookworm and *S. stercoralis* larvae. They are time consuming, require special equipment and dedicated, trained laboratory staff. Therefore, they are rarely used outside of specialized parasitological laboratories or specific research projects. [34]

According to *Marchi Blatt and Cantos, Kitvatanachai and Pipitgool, Arakaki et al., Glinz et al.* and *Machicado et al.* the Agar plate culture shows the highest sensitivity for the detection of *S. stercoralis* larvae. The Harada-Mori culture proved to give the highest yield in Hookworm infections whereas it shows only a low sensitivity in detecting a *S. stercoralis* infection. The Agar plate culture method has been recommended for epidemiological research questions. [14, 28, 29, 51, 52]

A real-time PCR technique to detect faecal *S. stercoralis* DNA applied by *Verweij et al.* showed a high sensitivity with 100% specificity. Compared to the Baermann culture technique the authors reported a 2-fold increase in the detection rate. [21]

Several recent studies for the diagnosis of *S. stercoralis* infection describe a high sensitivity of real-time PCR compared to the culture techniques.[21,

14

22, 53] A study by *Becker at al.* shows that the PCR method is the most sensitive method to detect *S. stercoralis* infections in comparison to stool microscopy techniques such as the Agar plate method and Baermann technique with which half of the infections would have been missed. The sensitivity of the Agar plate method, Baermann technique and real-time PCR for detection of *S. stercoralis* compared to a combination of all three diagnostic tests was 21.4%, 37.5% and 76.8%, respectively. [20]

To diagnose a *S. stercoralis* infection a study on returning travellers and immigrants in the Netherlands showed an increased detection rate by 0.8% with realtime PCR compared to an antigen assay and microscopy method. Microscopy and antigen assays of 2,591 stool samples showed a *S. stercoralis* infection in 0.1 % of the cases, as only three samples with a *S. stercoralis* larvae were detected using microscopy. Performing realtime PCR in total 21 stool samples showed a positive results. The study was performed over a period of 13 months. In total 2.591 stool samples were examined. [54]

The PCR method was claimed to have a sensitivity of 100% compared to the Agar-plate method in a study performed in Iran which suggests a higher efficiency in diagnosing an infection in comparison to the Agar plate method. [55] In a study by *Repetto et al.* a sensitivity of 100% of the PCR method is described. The superiority of the PCR is shown in comparison to other conventional methods as the Agar plate culture. Moreover, the study indicates that PCR shows positive results in the first tested stool sample whereas the Agar plate method required up to four samples for diagnosing an infection in some samples. One subgroup has been HIV positive patients. These results show the effectiveness of detecting *S. stercoralis* DNA with the PCR method in HIV positive patients.

Furthermore, the PCR results show a significant superiority for detecting an infection within asymptomatic patients compared to larvae detecting methods such as the Agar plate culture or the direct examination. The

absence of symptoms suggests a lower larvae load and infection with decreased intensity. This proves the efficacy of the PCR in diagnosing infections with low larvae intensity. [56]

Moreover, a study by *Marra et al.* published in 2010 proved the accuracy of the PCR method for the diagnosis of a *S. stercoralis* infection even with low intensity. In the study Lewis rats were experimentally infected with high, moderate and low larvae count. Their results showed that the PCR method is a sensitive procedure to detect *S. stercoralis* larvae even if only a light infection is present. [57]

Also, data enrolled in a study in Kenya by *Arndt et al.* implicate that PCR diagnoses infections of low intensity which may be undetectable by traditional methods. [19]

A study conducted in Cambodia with randomly collected faecal samples of asymptomatic school children showed a sensitivity of the PCR method of 88% for detecting a *S. stercoralis* infection [53]

A study by *O´Connell et al.* described the possibility of false positive results in the Baermann technique or Agar plate method through interpreting results as positive for a *S. stercoralis* infection although an actual Hookworm infection is present. In these cases it gets obvious that the realtime PCR as being the most specific and sensitive method shows negative results concerning a *S. stercoralis* infection. [58]

The difficulty of detecting a *S. stercoralis* infection in immunocompromised patients is described by *Gómez- Junyent et al.* The results of their study show that only the realtime PCR succeeds in diagnosing a *S. stercoralis* infection in immunocompromised individuals whereas serological testing and stool examination remain negative. The study suggests the realtime PCR being the best diagnostic tool to detect an infection within immunosuppressed patients. [59]

In contrast to the results of the above mentioned studies *Knopp et al.* showed a low level of sensitivity with only 17.4 % when comparing the PCR method to the Baermann technique. In this study stool samples of asymptomatic children with a median age of 5 years in Tanzania were examined with different methods to detect a *S. stercoralis* infection. However, a multiplex realtime PCR for a simultaneous detection of A. lumbricoides, *N. americanus, S. mansoni and S. stercoralis* DNA was used which could explain the low sensitivity value of the PCR method. Nevertheless, the specificity of the PCR method compared to the Baermann technique was significantly higher. (93.9% versus 78.4%) [60]

In a sero-epidemiological study in the Peruvian Amazon region the *S. stercoralis* antibody ELISA was found to have a sensitivity of 92% and a specificity of 94%. The negative predictive value was reported to be 98% making the ELISA an excellent screening tool for Strongyloidiasis [61].

There are recent studies which describe the ELISA for having a high sensitivity but lack of specificity since there are cross reactions with other helminth infections. Furthermore, there is the possibility of persisting titres of already cured infections. Therefore, higher positive results can occur with the ELISA method compared to direct detection methods. Nevertheless, the ELISA is described as a very successful tool for evaluating the effectiveness of a therapy. In general antibody titres decline after six to twelve months after successful treatment. In returning travellers the ELISA is therefore a suitable method for therapy control. Considering a high risk of reinfection persisting antibody titres can be interpreted as a therapy failure or reinfection [46, 62, 63]

A lack of sensitivity of the ELISA was detected during tests of immunocompromised patients as presented in a study by *Luvira et al.* [64]. In the study conducted in Bangkok/ Thailand, 135 adults with various immunocompromising conditions underwent an Ig G ELISA as well as a stool collection. Faecal samples were screened with a simple smear, the

Formalin Ether Concentration Technique, as well as the Agar Plate Method. Results of the study showed a prevalence rate of Strongyloidiasis of 5% using the stool concentration technique, 5.4 % in the Ig G ELISA and 6.7 % using the Agar Plate method. The sensitivity of the Ig G ELISA was only 42.9%. In immunocompromised patients the antibody response to *S. stercoralis* seems unpredictable, therefore.

3.8   *S. stercoralis* and HIV-Co-infection

S. stercoralis infection was significantly more prevalent among HIV-infected patients in a study conducted in Brazil by *Marchi Blatt and Cantos*. The authors concluded that HIV-positive patients were 5 times more susceptible to *S. stercoralis* infection than HIV-negative controls.[14] Despite the documented higher rate of *S. stercoralis* infection in HIV-positive individuals, HIV infection alone is not the greatest risk factor for the development of a hyperinfection syndrome. [65]

It must be pointed out that disseminated Strongyloidiasis was on the list of opportunistic infections. No case of disseminated Strongyloidiasis was found in necropsy studies on HIV-infected patients from Brazil and Africa. Although there is a possibility that cases of disseminated Strongyloidiasis might have been unrecognized as clinical monitoring in the parts of the world in which *S. stercoralis* is endemic might have been less broad and accurate than in more developed countries the CDC and WHO removed disseminated Strongyloidiasis from the list of opportunistic infections in 1987. [66]

Conditions showing an impairment of the cell-mediated immunity have been identified as risk factors for the hyperinfection syndrome and disseminated disease. Among them the two conditions with the strongest association are an infection with the human T-lymphotropic virus type 1 (HTLV-1) [2] and the use of corticosteroids. [10] An explanation for this clinical observation was published by *Siegel and Simon*. They concluded

that the main immunosuppressive effect of HIV-infection is the cellular immune deficiency caused by the destruction of CD4+ lymphocytes. Within the CD4+ cell population a relatively greater decline in the activity of type 1 T helper cells (Th1) than type 2 T helper cells (Th2) has been found. Advanced AIDS is usually associated with a profound loss of Th1 immune activity and decline of the proinflammatory cytokine levels (IFN-γ, IL-2, TNF-α). In contrast the Th2 cells -which are more active in mediating humoral immunity- are less affected and the Th2 mediated cytokine profiles (IL-4, IL-5, IL-10, and IL-13) show little change. The levels of Th2 mediated cytokines IL-4 and IL-10 have been found to be higher in HIV-infected patients (27 [66]). This could result in a conserved Th2-mediated response to helminthic infections. IL-4 and IL-5 stimulate IgE-production which causes mast cell degranulation and stimulates mucous production by intestinal goblet cells. In addition, IL-5 acts as eosinophil colony stimulating factor and stimulates the migration and activation of eosinophils. In general eosinophils play an important role in the human defence against parasitic helminthic infections.

IgE antibodies are commonly elevated in patients with helminthic infections including HIV-infected individuals. In patients with more advanced HIV-infections with low CD4-cell counts even higher IgE levels were found. It has been suggested that HIV gp120 acts as an allergen which is stimulating the production of IgE. [66]

In contrast to the widely preserved Th2 activity in HIV-infection a HTLV-1 infection causes an immunologic shift to a Th1 cell type response with increased levels of IFN-γ and reduced levels of IL4 and IL5. This results in lower IgE levels, lower peripheral eosinophil counts and reduced response to anthelminthic treatment. [67] These findings may explain why HTLV-1 infection is a significant risk factor for the *S. stercoralis* hyperinfection syndrome and disseminated disease, while HIV-infection without other immunosuppressive factors is not. So far unexplained is the observation

of a higher prevalence of *S. stercoralis* infection in HIV-infected individuals. [14, 68, 69] *Siegel and Simon* suggest a higher susceptibility to infection due to chronic immune activation.[66]

Animal models indicate that dissemination of *S. stercoralis* in the immunocompromised host is promoted by a switch towards direct development of rhabditiform larvae into infective filariform L3 larvae. [70] A clinical study of *Viney et al.* in Uganda showed in fact an opposite effect with a higher rate of direct development in higher CD4-levels. [65] This observation is consistent with the unexpected low incidence of hyperinfection syndrome in HIV-infected adults. [70]

Thus, a high dose corticosteroid treatment in case of an immune reconstitution syndrome after the start of ART causes the risk of developing a *S. stercoralis* hyperinfection syndrome with high mortality rates. Under these circumstances HIV positive patients are exposed to a substantial at risk.[8, 71, 72]

4. Methodology and Patients

4.1 Study location, Study population and Ethics

4.1.1 Location of the Study

The study was conducted in the Care and Treatment Centre (CTC) and the Medical Department of the Bugando Medical Centre (BMC) in Mwanza, Tanzania. The BMC is the referral hospital for the Lake and Western Zones of the United Republic of Tanzania serving a catchment area with a population of about 13 million people. The CTC currently takes care of more than 12.000 HIV-positive patients. More than a hundred patients are seen per day. The analysis of the ward logs shows that on average about 30% of all admissions to the medical department are due to HIV-associated medical conditions. The Bugando Medical Centre serves as a referral hospital with a huge catchment area and is attracting patients for specialized care from the urban as well as from rural areas. It

can therefore be expected that the study population has a mixed background concerning socioeconomic status and living conditions.[73]

The joint institutional review board of the Bugando Medical Center / Catholic University of Health and Allied Sciences (CUHAS) approved the study proposal. HIV positive adult patients were randomly included and asked to provide a blood and a faecal sample. In addition, an in- depth interview was conducted to get viable information on the medical background and living conditions. The Bugando Medical Centre served as a favourable place for this study since the catchment area of the hospital included HIV positive patients from rural as well as from urban settings.

Figure 6: Catchment area of the Bugando Medical Center (marked areas)
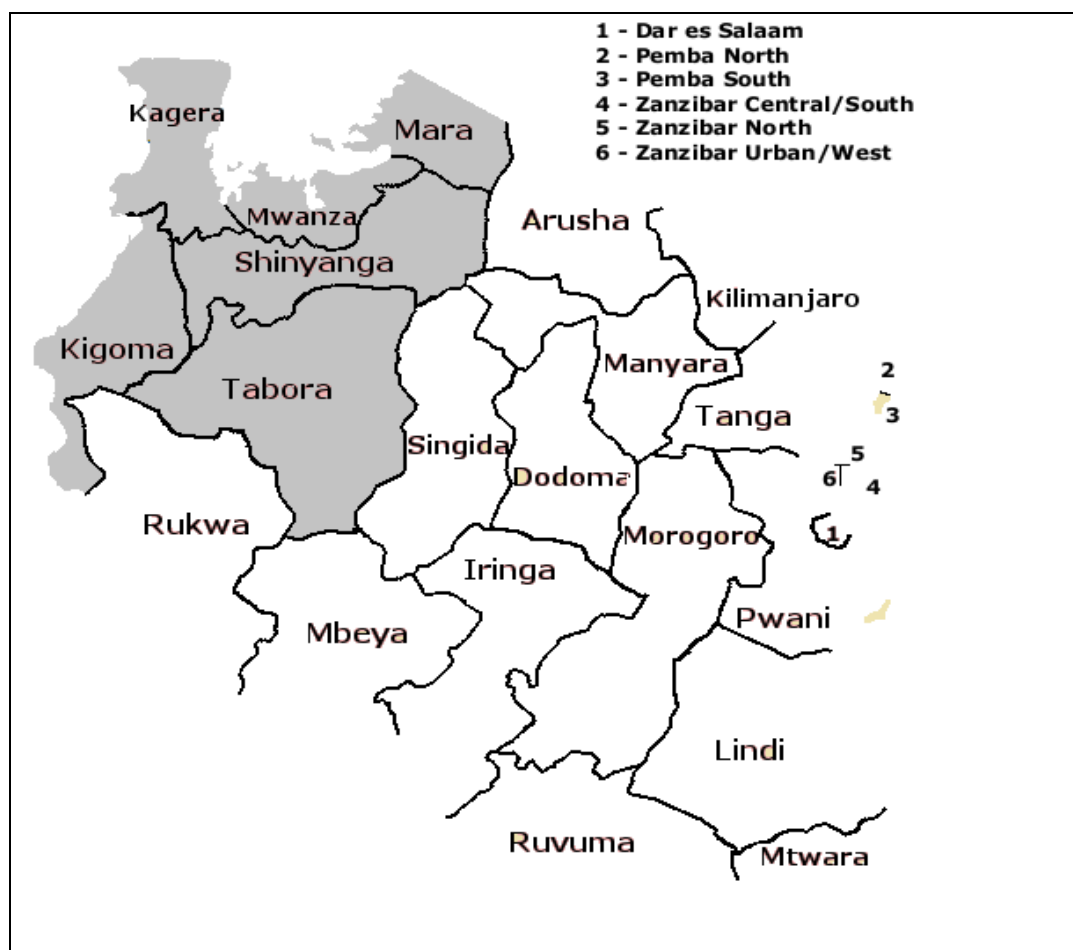
Modified from source: www.weltkarte.com

4.1.2 Study population

The samples were randomly selected from male and female HIV positive patients older than 18 years and regularly attending the CTC as well as from patients admitted to the medical wards of the BMC. Patients who had received standard anthelminthic treatment (400 mg albendazole once) were not excluded from the study since the commonly used deworming regimen with a single dose of albendazole or praziquantel is assumed to have little impact on *S. stercoralis* infection.

According to the study protocol patients who had received anthelminthic treatment with either ivermectin (2x200 µg/ kg of body weight) or high dose albendazole (400 mg bid for 7 days) within the past six months were excluded from the study. The history of anthelminthic pre-treatment was recorded in the questionnaire. Moreover, the informed consent had to be signed.

4.1.3. Ethics

The study proposal was presented and discussed in the joint institutional and ethical review board of the Bugando Medical Center /Catholic University of Health and Allied Sciences and the permission was obtained to conduct the study.

The study was conducted in the following way: The patients were thoroughly counselled about the purpose of the study and after their written consent they were asked to provide a serum sample for antibody testing. The blood samples were taken by experienced hospital personal using sterile, disposable material. There were only minimal expected side effects like small visible haematoma at the site of vein puncture. Providing a stool sample did not bear any risk for the participant.

The results of the culture techniques were made available within 1 week after the enrolment of the patient. Positive results for *S. stercoralis* infection were reported to the wards of the CTC immediately and participants were

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offered a free treatment with albendazole 400 mg bid for 7 days as ivermectin was not licensed and not available in Tanzania for this purpose during the time of the study. In case of other intestinal parasitic diseases detected by faecal examination appropriate therapy was prescribed according to published guidelines.[74]

No other than the tests documented above were done. Remaining samples will be destroyed latest five years after the end of the enrolment period.

The study data will be presented on national and international conferences and published in medical journals as soon as the data analysis is completed. Any publications resulting from this study will be done in mutual agreement and according to the individual contribution of the investigators

4.2 Data collection and statistics

4.2.1 Questionnaire and selection of patients

An in- depth questionnaire was elaborated and translated to Kiswahili as presented in the annex. Each patient was asked about his / her profession, living conditions and access and use of sanitation. With the help of a senior nurse the personal data with emphasis on socioeconomic status, level of education, epidemiological risk factors such as sanitation and access to water and previous courses of anthelminthic treatment were collected. In addition, data indicating a higher risk for gaining chronic *S. stercoralis* infections were retrieved from the patients' records including the history of additional chronic illnesses, previous opportunistic infections, latest CD 4-cell count, lowest CD 4-cell count as well as data on antiretroviral therapy and the use of immunosuppressive drugs.

Participants were approached by a senior nurse and counselled about the purpose of the study and the personal benefit. Participation was voluntary, and no remuneration was paid for participation. If the patient gave his informed consent, he was asked to complete the in-depth questionnaire.

The questionnaire was performed with all participants of the study and evaluated by calculating the odds ratio in to verify risk factors for gaining a *S. stercoralis* infection.

After the answering the questionnaire the participants were asked to donate a blood sample and received 2 stool sampling containers labelled with the study number.

The efficacy of different treatment regimens was considered in the study design. Patients with ivermectin treatment or high dose albendazole were excluded from the study. Single dose albendazole 400mg was considered as insufficient for the treatment of *S. stercoralis* and patients could be included. [49]

4.2.2 Sample size and analysis

The study has been designed to deliver data on the prevalence of *S. stercoralis* infection in patients with HIV-infection. For the estimation of the required sample size assumptions of the likely prevalence level had to be made based on the best available prevalence data from the region. *Mariam et al. 2008.* found a prevalence rate among HIV positive adults of 11,5% in Ethiopia. A similar study by *Knopp et al.* found a prevalence rate of 12% among the rural and periurban population in Zanzibar but they did not differentiate between HIV-positive and HIV-negative participants. [15, 27] Assumption: Based on the results of the two studies an expected prevalence rate of 12% was assumed. Based on this assumption the sample size was calculated according to a formula which has been developed and published by Leslie Kish, an American statistician in the 1960ies as follows:[75]

$$n = \frac{Z^2 P(1-P)}{d^2}$$

Z= level of confidence. For the conventional level of confidence of 95%, the Z value is 1.96.

n = sample size

p = expected prevalence (in proportion of one)

d = precision (in proportion of one)

N = 1.96^2 * 0.12 (1-0.12) / 0.05^2

N = 3.8416 * 0.1056 / 0.0025

N = 162

According to the formula at least 162 samples had to be included into the study to reveal the prevalence of *S. stercoralis* in the North Western Lake Region of the United Republic of Tanzania.

To overcome the problem of scanty data on the regional prevalence of *S. stercoralis* infection in HIV infected patients the aim was to include > 245 patients. In the end 278 patients participated.

Additional data were retrieved from the patients' files concerning comorbidity and immune status. The planned enrolment period was 2 months.

All personal data were kept confidential and were accessible only to the staff directly involved in data entry and analyses. Personal data were only used to identify patients eligible for anthelminthic treatment. Data were entered into a data base using Microsoft Excel on password protected computer systems.

Data entry was done using Microsoft Excel 2010 (Microsoft Corporation) and analysed using IBM SPSS Statistics version 21.0. Bivariate correlation analysis was done and correlation coefficients (Pearson product-moment

correlation coefficient) calculated. P value P < 0.05 was considered being significant at a 95 % confidence interval.

4.3 Laboratory analysis

Fresh faecal samples were used to perform the Agar plate culture and modified Harada Mori culture technique right on the spot at the BMC Clinical Laboratory.

For preservation an aliquot of each sample was transferred into 2 ml cryovials. One of the two cryovials was filled up with 70% Ethanol for performing the realtime PCR. The second one was preserved with 10% Formalin for microscopy after concentration using the Faecal Parasite Concentrator method (Evergreen Scientific). These two investigations were performed at the Medical Mission Hospital / Institute, Wuerzburg, after shipment of the samples

The blood sample was centrifugated and the serum, was carefully transferred into cryovials and stored at minus 20°C. *Strongyloides* antibody testing was performed after shipment of the specimens to the Bernhard-Nocht-Institute in Hamburg/ Germany as it is currently not available in Tanzania. An in house ELISA technique was used for this purpose. The following four methods were used for the stool analyses for *S. stercoralis* larvae.

4.3.1 Agar Plate Culture

The Agar plate culture was applied as described by *Koga et al*. The culture plates were prepared with Mueller-Hinton agar using disposable Petri dishes of 9 cm diameter. The Petri dishes were sealed with parafilm M and approximately two g of faeces were applied per culture plate. The culture plates were kept at ambient room temperature (around 28°C) and evaluated after 24, 48 and 72 hours using a Novex stereo microscope. [76]

4.3.2 Modified Harada Mori Culture

The Harada-Mori culture technique was applied with the following modifications: 50 ml polypropylene centrifuge tubes were used instead of 15 ml tubes allowing the use of a larger stool quantity. The conical end of the centrifuge tube was cut off and a 10 mm microscopic cover glass was glued to the end of the tube with a special cyanoacrylate adhesive (see figure 7). This allowed the use of an inverted microscope for examination (see picture 1).

For performing the Harada- Mori culture technique each conical tube was filled with ten ml of normal tap water. A strip of chromatography paper was inserted into the tube without reaching to the bottom. By this means a humidity gradient along the strip of paper was created. On the uncovered end of the paper about 0.5g of fresh faeces were applied. The tubes, incubating the cultures at room temperature (28°C), were examined with an inverted microscope (Hund/Wetzlar) every 24 hours for six days. In case nematode larvae were detectable it was planned to collect them from the Harada-Mori culture tube for examination at high power magnification after fixation with ethanol. High power magnification allows to differentiate between *S. stercoralis* and Hookworm larvae.

Figure 7: The modified Harada Mori technique:

Source: own figure prepared with Microsoft paint creation
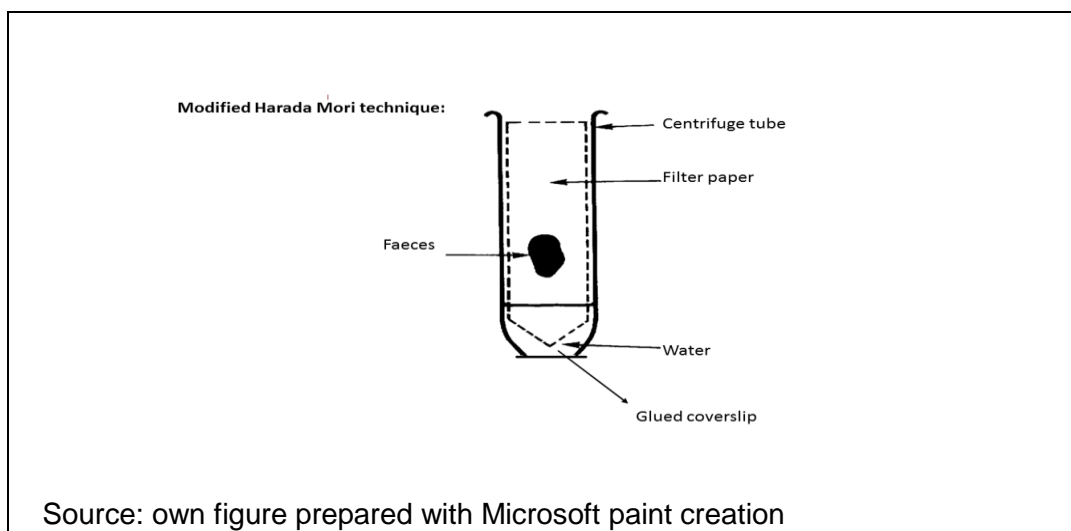
Photograph 1: Analysis of the modified Harada Mori culture tubes with an inverted microscope

Own source

Photograph 2: Samples of Agar plate method and the modified Harada Mori method

Own source

4.3.3 Faecal Concentration technique

The Faecal Parasite Concentration (FPC) technique by Evergreen Scientific was used since it is known for producing the cleanest and best slides to read as shown in a study by *Perry et al.* who tested five different stool concentration systems with known parasites. All five concentration techniques were similar in the detection rate. Compared to microscopy of fresh unconcentrated stool specimen the five concentration techniques were more effective in detecting parasites.[77]

In this study microscopy using the FPC technique was done from a formalin preserved specimen. The main intention to use a concentration technique was for the differential diagnosis of other intestinal parasites. It is well known that concentration techniques have a low sensitivity for detecting *S. stercoralis* larvae. [14]

The FPC method was performed according to the protocol of the manufacturer (Evergreen Scientific). The specimen preserved in 10% formalin were transferred to the tubes and plastic trays provided by the commercial FPC kit. Into each flat-bottomed tube 9 ml of 10% formalin were filled, three spoonsful of preserved stool were added and mixed thoroughly. In the next step three drops of Triton X- 100 were added as well as 3 ml of ethyl acetate. After checking that the provided 15ml tubes are safely connected to the FPC filter the vent straw in the filter unit was pulled out about for one inch. Then the FPC filter was tightly fitted on the flat-bottomed tubes which contained the faecal specimen. Thereafter, the specimen was shaken and poured through the filter into the 15ml conical tubes while the conical end pointed downwards. The flat-bottomed tubes including the FPC filter was then discarded in appropriate manner. Centrifugation of the 15ml tubes followed at 500 rpm/g for 10 minutes.
After centrifugation an arrangement of the specimen in four layers is visible.

With a cotton applicator stick the debris was cleared from the rim in order to pour off the supernatant fluid.

Remaining debris and ethyl acetate were removed from the rim by using a cotton tipped applicator.

After pouring off the supernatant only the sediment on the bottom of the tube remains and is mixed with three drops of 10 % formalin. Coverslip slides were prepared for microscopy using a transfer pipet applying the sediment from the bottom of the tubes to the slides. These were systematically screened with a Zeiss Standard® light microscope at 100x and 400x magnification.

4.3.4 DNA Isolation

For DNA isolation a commercial QIAmp DNA stool MiniKit by QIAGEN, Hilden/ Germany was used. Extraction of genomic DNA was performed by using approximately 1g stool of the 70% ethanol preserved stool samples. The specimen was adjusted with 3 ml ether. After shaking the suspension, it was centrifuged at 1000 rpm/g for two minutes. Extraction of the DNA could begin after washing the pellet with distilled water. According to the instruction 1,4 ml Automated Lab Solution (ASL), which is a stool lysis buffer, was added to each stool sample and vortexed for roughly one minute until an entirely homogenization was achieved. This guaranteed a maximum DNA concentration in the final eluate.

In the next step the suspension was heated for 5 minutes at 70° C. Another vortexing with 3000 rpm of about 15 seconds followed and centrifugation of one minute to sediment all the stool particles. 1.2 ml of the supernatant was transferred into a new 2 ml microcentrifuge tube. To each sample of supernatant one InhibitEX tablet was added. Again, the sample tube was vortexed with 3000 rpm to disperse the reagent tablet completely.

An incubation period of one minute at room temperature ensured that potential inhibitors adsorbed to the InhibitEX matrix. A complete attachment of inhibitors to the InhibitEX matrix was reached through centrifuging the sample for three minutes.

All the supernatant was pipetted in a new 1.5 ml micro centrifuge tube. Another centrifugation of three minutes with 3000 rpm followed.

In the next step 15 µl of Proteinkinase K was filled into a new 1.5 ml micro centrifuge tube, to which 200 µl of the supernatant from the step before was added. Proteinkinase K is a protease isolated from a fungus and possesses a high specific activity, remaining stable over a wide range of temperatures and pH values.

The sample was incubated at 70°C for 10 minutes and in the following step 200 µl of 96-100% ethanol was added and mixed by vortexing with 3000 rpm.

In the next part of the process the whole lysate from the step before was filled into a QIAamp spin column. This was placed into a new labelled 2 ml collection tube and centrifuged for one minute. The QIAamp spin column was put into another new labelled 2 ml collection tube. The tube from before containing the filtrate was completely discarded with its content.

The QIAamp spin column was opened and 500 µl Buffer AW 1 were added which is used to denature the proteins so a passing through the filter is guaranteed. A centrifugation of one minute followed. The QIAamp spin column was placed in a new labelled 2 ml collection tube. The tube containing the filtrate was ejected.

Then 500 µl Buffer AW 2 was added. This buffer is used to wash out the remaining salts. The tube was closed and again centrifuged for three minutes. Again, the tube containing the filtrate was discarded. As recommended in the instruction's manual the QIAamp spin column was placed in a new 2 ml collection tube and centrifuged for one minute. This increased the probability that all possible remnants of Buffer AW 2 were eliminated.

The QIAamp spin column was transferred into a new labelled 1.5 ml microcentrifuge tube. After opening the QIAamp spin column 200 µl Buffer AE was added directly onto the QIAamp membrane. The tube was closed

and an incubation for one minute at room temperature followed. Subsequently the tube was centrifuged for one minute to eluate the DNA.

4.3.5 DNA quantitation

In order to ensure a sufficient DNA concentration in the pellets for performing realtime PCR ten samples were randomly selected. These ten samples were revised with the Qubit®dsDNA BR (Broad Range) Assay Kit to visualize the DNA quantity.

This was performed as follows:

Ten 0.5mL tubes were set up as well as two extra tubes for the two Qubit dsDNA BR Assay standards. All tubes were labelled carefully.

In the next step the Qubit working solution was prepared by diluting the Qubit dsDNA BR reagent in the dedicated Buffer to a concentration of 1:200 in a clean plastic tube.

To the two labelled tubes used as standard 190 µL of Qubit working solution were added. To each tube exactly 10 µL of each Qubit standard were filled and then mixed through vortexing with 3000 rpm for 2-3 seconds.

Into the individual assay tubes containing the extracted DNA pellet Qubit working solution was added so that the final volume reached 200 µL. Vortexing with 3000 rpm for 2-3- seconds followed as well as an incubation of the assay tubes at room temperature for 2 minutes.

After calibration of the Qubit 2.0 fluorimeter with the two Standards the ten different samples were inserted into the sample chamber and read. A calibration was performed after reading each sample.

On the screen of the fluorimeter the results appeared and could be noted. The displayed results on the screen are equal to the DNA concentration after diluting the sample within the sample tube. In order to verify the concentration of the original sample the calculation had to be done for each result as follows:

Concentration of the sample = QF value × $\frac{200}{x}$

QF value = result given on the screen of 2.0 Fluorimeter

X = microliters of sample added to the tubes (190 µl)

The result of formula is given in µg/ml.

4.3.6 Realtime PCR

The real-time-PCR for *S. stercoralis* was performed according to the method described by *Verweij et al.* (23 [21]). *S. stercoralis* specific primers from the 18S rRNA gene sequence (GenBank accession no. AF 279916) in a concentration of 250nmol and hydrolysis probe in a concentration of 76nmol were used. (Product from Eurofins MWG Operon). Concentrations were optimized for the PCR machine. As internal positive control for the DNA amplification served the IPC Mix and IPC DNA (produced by Applied Biosystems/ Life Technologies).

In total 52 batches were mixed as the core material for the analysis consisting of the following:

- 299 µl distilled water
- 650 µl of Master Mix (produced by Applied Biosystems/ Life Technologies)3.130 µl IPC mix26 µl IPC DNA added with 65 µl PP Mix (Primer probe).

Amplification was done using the Step One PCR Machine (from Applied Biosystem) fitting in total 48 probes.

The **negative control** consisted of 4 probes as follows:

- two samples which were mixed using 22.5µl of the core material as described before adding 2.5µl IPC Blocking,
- two samples with 22.5µl of the core material adding 2.5µl of distilled water.

The **positive control** consisted of 1 probe as follows:

- 22.5µl of the core probe adding 2.5µl of *S. stercoralis* DNA which was allocated by the Swiss Tropical and Public Health Institute.

The remaining 43 samples were filled with 22.5µl of the core probe adding 2.5µl of the eluted DNA. Each cap therefore had a volume of 25µl.

Amplification detection and data analyses were executed using the **Step One Software** and consisted of the following 5 steps:

1. 30 seconds at 60°C: Pre PCR read
2. 10 minutes at 95°C: Holding Stage
3. 15 seconds at 95 °C: Breaking of double stranded DNA ⎤
4. 1 minute at 60°C: Attachment of Primer and elongation ⎦ Cycling stage of 50 cycles
5. 30 seconds at 60°C: Post PCR read

Table 1: Realtime PCR: Oligonucleotid primers and detection probes

Target (GenBank accession no.) Oligonucleotide name	Oligonucleotide sequence	Expected product size
S. stercoralis 18S rRNA gene (AF279916)		
Stro 18S- 1530F	5´-GAATTCCAATAAACGTAAGTCATTAGC-3´	101bp
Stro 18S- 1630R	5´-TGCCTCTGGATATTGCTCAGTTC-3´	
Stro 18S- 1586T	FAM-5´ACACACCGGCCGTCGCTGC-3´-BHQ1	

4.3.7 In house ELISA Technique

Serum analysis was done after shipment of the samples to a collaborative laboratory of the Bernhard Notch Institute in Hamburg/Germany with the following method:

- An in-house ELISA developed by the Bernhard Notch Institute for tropical medicine based on *S. ratti* antigens. *S. ratti* is hereby kept in a laboratory cycle.

- The larvae gained from rat faeces were washed in PBS and afterwards lyophilized, grinded and re-suspended in distilled water. A sonication with ultrasound in pulse mode follows for eight times each for three seconds. This is in total repeated three times.

- In the next step an ultracentrifugation (30.000 RPM) is done.

- The supernatant, an aqueous extract is used in a concentration of 0.5 µg per well in the ELISA- plate. Annealing of the antigens takes place over night incubated at room temperature.

- A washing with PBS/ 0.5% TWEEN and blockade over one hour with PBS/ 3% skim milk powder at room temperature followed. Afterwards the washing in PBS/ 0.05% TWEEN is repeated.

- The serum of the patients was diluted with PBS/ 3% skim milk powder in a concentration of 1:100 and was incubated for 45 min at room temperature and then washed with PBS/ 0,05% TWEEN.

- The last step is the incubation with anti- human- Ig G- alkaline phosphatase- conjugate, which is obtained from goats in a concentration of 1:10000, for 45 minutes at room temperature.

- After P-nitro-phenyl phosphate is added and a photometric measurement at 410 nm with a reference filter at 490 nm is done the antibody titres can be read.

4.4. Limitations of the Study

According to *Knopp et al.* and *Hirata et al.* repeated sampling may significantly increase the yield of intestinal parasites. This also applies to Strongyloidiasis as described by *Khieu et al. Hirata et al.* reported a two-fold increase in the detection rate after examination of three specimens per patient. However repeated sampling was not feasible in this study since outpatients were included in the study design making repeated

sampling logistically impossible. Instead of the use of multiple examinations we used a combination of tests including stool PCR and serum antibody testing by ELISA. [32] [4] [78]

5. Results

5.1. Risk profile of the Patients to acquire Strongyloides infection

In total 278 HIV positive patients participated in the study 76.6% were women, 23.4% were men. The study population was aged from 18 to 83 years. The median age was 43 years with a standard deviation of 10.1.

A standardized questionnaire (see appendix) was used to assess risk factors involved in the transmission of *S. stercoralis* infection. The risk factors were categorized into 3 main groups: profession, living conditions and access to sanitation.

Profession:

> 94 % of all participants claimed being farmers and only 5% stated to have other professions than farmer. 1% did not give a statement at all.

Living conditions:

> Living condition differencing between urban or rural.
>
> Living in an urban condition was named by 90% of all questioned patients and only 10% stated living in a rural area of Tanzania.

Sanitation:

> Standard of sanitation differencing between having a flush toilet or latrine as well as differencing between having accesses to tap water.
>
> A high amount declared to have access to tap water at home with a percentage of 93% whereas only 7 % named not to have access to tap water. 59% of the participants had a flushed toilet at home and 40% stated using a latrine as one percent did not give a statement.

Wearing shoes:

This risk factor for the study population was taken into account revealing that 62% of the patients stated to wear shoes often while 38% stated to always wear shoes.

Deworming therapy:

Deworming during the last twelve months with treatment of ivermectin or a high dose albendazole would exclude the patients in participating in the study. As ivermectin was not accredited in the National Guidelines in Tanzania as deworming therapy during the clinical trials no participant had received ivermectin as deworming therapy within the past twelve months. However, 31 participants received a single dose of albendazole (200mg or 400mg) within the last 12 months and five patients received a single dose of mebendazol within the past twelve months.

Patients health records:

All participants received antiviral therapy while there were thirty out of 278 HIV positive patients who had a CD 4 cell count below 200 / µl. But none of these had a positive result in the realtime PCR for *S. stercoralis*. Nine patients received immunosuppressive therapy, as in corticosteroid treatment. Dosage and duration of treatment was not documented in the patients' health record. Among these patients two had positive results in the PCR. According to the medical records seventy patients had a medical history of tuberculosis, one patient of a *Pneumocystis jirovecii* infection and one patient of a Cryptococcus meningitis. In addition, the medical records showed five patients suffering from allergic asthma and one patient from diabetes mellitus.

In order to assess the influence of social and economic factors for the risk of gaining *S. stercoralis* infections the Odds Ratio (OR) was calculated.

The OR demonstrates the likelihood or the odds of an outcome which appears if a certain exposure is given in comparison to the odds of the

outcome with the absence of this exposure. In this study the OR was calculated in order to verify whether the factors collected with the in depth questionnaire can be considered as risk factors for gaining a *S. stercoralis* infection. The results of the OR are interpreted as follows:

- OR = 1 shows that there is no likelihood that this factor has an influence on the outcome or infection,

- OR > 1: shows that there is a high likelihood that this factor is associated or has an influence with the outcome for gaining an infection,

- OR < 1: shows that the exposure or this factor has a low association or influence with the outcome for gaining an infection. [79]

The reference group for comparison is put in the parentheses for the compared factors as shown in the following table 2.

The results show an OR greater than 1 for the factors sanitation here the availability of a latrine or flush toilet and profession, here farmers, which indicate a high association with the outcome or likelihood of gaining an infection. The other socio-economic factors show little relation to the exposure. However, the table demonstrates that none of the investigated risk factors showed a statistically significant association with gaining a *S. stercoralis* infection since the p levels are high. [79, 80]

Table 2: Calculated odds ratio

| Variables	Risk factors for gaining a *Strongyloides stercoralis* infection	
	Odds Ratio (95% CI)	*P*
Gender (Ref = unifected male) Female	0.549 (0.195- 1.805)	0.354
Profession (Ref = uninfected Other than farmer)	1.267 (0.155-10.340)	0.825
Water access (Ref = uninfected no tap water) Tap water	0.416 (0.86- 2.012)	0.262
Living condition (Ref = uninfected rural) Urban	0.605 (0.182- 2.864)	0.522
Deworming therapy (within last 12 months) (Ref = uninfected no therapy) Yes	0.627 (0.136- 2.886)	0.546
Sanitation (Ref= uninfected flush toilet) Latrine	1.118 (0.377- 3.316)	0.841
Wearing shoes (Ref= uninfected always) Often	0.820 (0.276- 2.433)	0.72

Own source

5.2 Comparisons of the applied methods

5.2.1 Prevalence analysis

The following figure shows the prevalence rates of *S. stercoralis* identified with the applied different methods.

Figure 8: Prevalence of *S. stercoralis* infection within different diagnostic methods
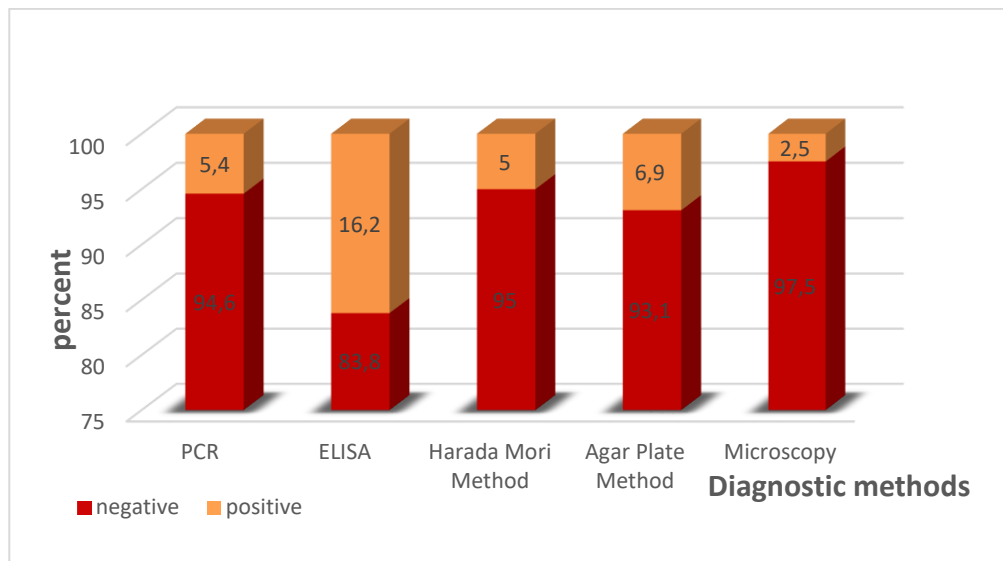
Own source calculated with PASW Statistic creation

The real-time PCR was positive in fifteen of 277 cases, -one sample could not be used due to a lack of feaces material-, resulting in a prevalence of a current *S. stercoralis* infection of 5.4%.

Elevated antibody titres in the in-house ELISA indicating a *S. stercoralis* infection were positive in 45 of 277 cases, as one sample was missing and therefore showed a percentage of 16.2%.

The evaluation of the 278 samples prepared with the modified Harada Mori technique assessed already before the third day, 48 hours after preparation, showed a positive larvae yield only in one case resulting in an estimated prevalence rate of 0.4%; the evaluation of the samples after 120 hours was positive in 13 out of 259 cases resulting in a total prevalence rate of 5.0%. This late screening of the samples caused a loss of 6.8 %.

Hence, 19 out of the 278 samples could not be analysed under the inverted microscope due to sedimentation of faecal particles. The following pictures 3 and 4 made with the inverted Hund/ Wetzlar microscope show *S. stercoralis* larvae in a sample prepared with the Modified Harada Mori technique.

Photograph 3: Anterior part of a *S. stercoralis*

Own source

Photograph 4: The *S. Stercoralis*

Own source

After incubation of 24 hours the Agar plate method showed a positive larvae yield in 6 out of 278 cases resulting in a prevalence rate of *S. stercoralis* larvae of 2.2%. An increasing incubation period of 48 hours led to the detection of larvae in 19 cases out of the 277 samples. One sample had to be rejected due to plate contamination. This resulted in a total prevalence rate of 6.9 %. A further increase of the incubation time up to 72 hours did not lead to a further increased detection rate.

The following microscopically pictures taken with the stereo-microscope shows the typical whipped like spoors of *S. stercoralis* larvae as described by *Ines Ede* and a *S. stercoralis* larvae at high power magnification. [81]

Photograph 5: Whipped like spoors of S. stercoralis larvae
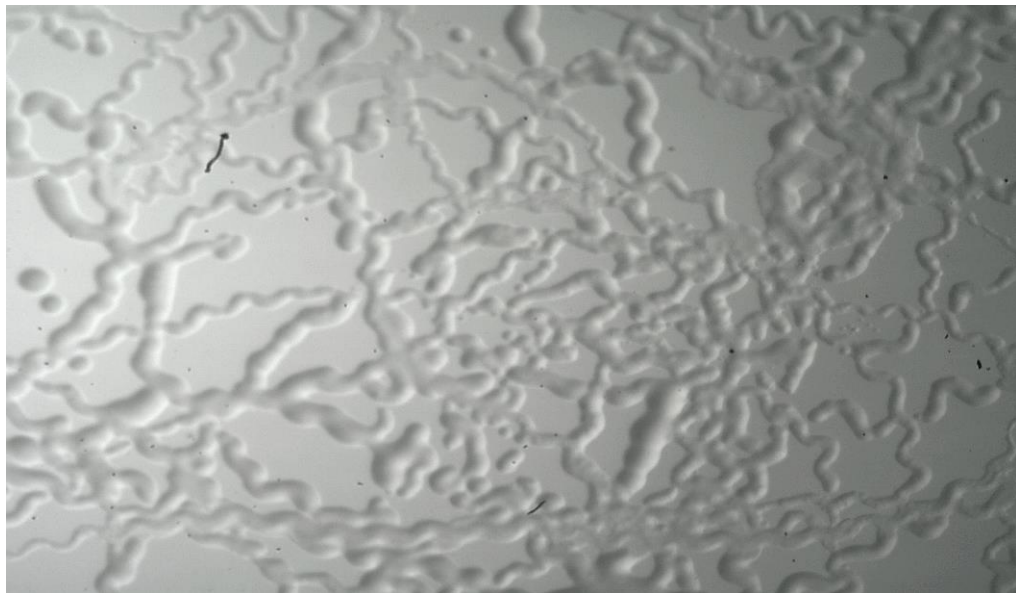
Own source

Photograph 6: S. stercoralis larvae.

Own source

Performing microscopy after processing the formalin preserved faeces samples with the FPC method showed a wide variety of intestinal parasites in the stool samples. Positive results of *S. stercoralis* larvae could be observed in seven of 278 cases resulting in a prevalence rate of 2.5 %.

In 20 cases *Schistosoma mansoni* eggs were found. 1 sample showed an *Ascaris lumbricoides* egg and 1 sample showed an *Entamoeba coli* as well as a *Jodamoeba bütschlii* cyst. 3 samples showed a Hookworm egg. 1 sample was considered positive in the Agar plate method after an incubation period of 48 hours but was negative using the PCR method. Of the two samples with a positive Hookworm egg both showed negative results in the realtime PCR and were claimed as positive in the Harada Mori method evaluating results on day 6.

Photograph 7: Hookworm egg

Source: *https://syllabus.med.unc.edu/ahs/clsc460L/00084.htm*
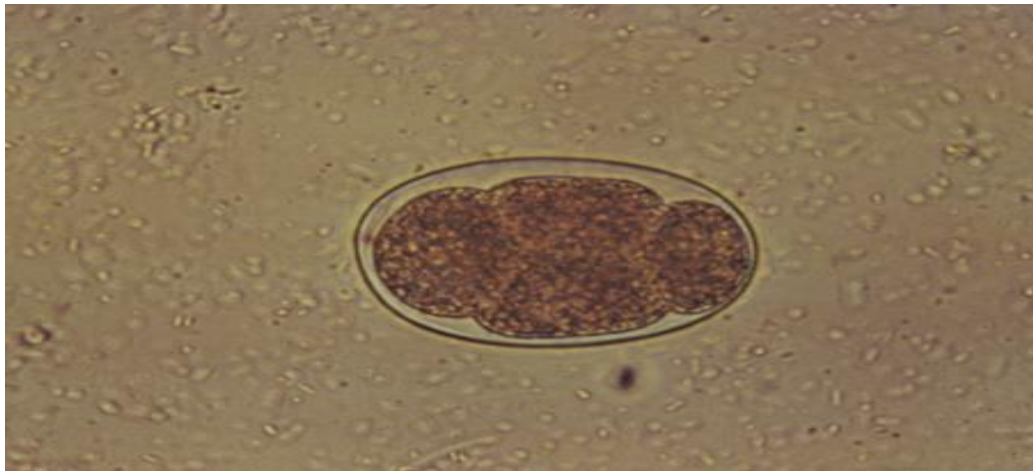
5.2.2 Specificity and sensitivity

According to the literature there seems to be a common consensus among the researchers that the PCR provides the best possible results for the prevalence. [53, 56]

Hence, to ENSURE that a sufficient amount of DNA was eluted through the commercial QIAmp DNA stool MiniKit by QIAGEN, Hilden/Germany for performing a realtime PCR, a DNA quantification was performed and verified with the Qubit®dsDNA BR (Broad Range) Assay Kit as described before.

For the test, ten different samples were chosen randomly for the DNA concentration analysis. The chosen samples had the case numbers 255, 203, 151, 189, 263, 253, 206, 199, 262, 226. The results of the DNA concentration tests are shown in the following table 3.

The results show a wide range of DNA concentration between 1.01 µg/ml and 22.80 µg/ml. Despite this wide range, these levels however can be considered as sufficient nucleic acids levels for operating the realtime PCR since a positive prevalence could be found in case number 262 with a rather low DNA concentration.

Table 3: DNA concentration in μg/ml by case

Case Number	DNA concentration μg/ml
255	22,80
203	14,10
151	9,00
189	7,02
263	2,58
253	2,42
206	1,97
199	1,86
262	1,20
226	1,01

Own source

The PCR was considered as the "Gold standard", as studies describe a sensitivity of 100%. [53, 56]

Sensitivity, specificity, negative predictive value and positive predictive value of each method were calculated using PASW Statistic 18.

Hereby, the sensitivity of a test represents the ability of the method to detect a true positive result whereas the specificity indicates the ability to exclude a true negative. Sensitivity and specificity are characteristics of the applied test or method and do not depend on the prevalence of the disease in the population tested. Often an inversely relation between specificity and sensitivity occurs. Whereas positive and negative predictive values rely on the actual prevalence of the disease. The positive predictive value increases as the infection prevalence increases. The higher the positive predictive value is the higher is the reliability that positive tested results are truly positive. On the other hand, the negative predictive value decreases as the prevalence of an infection within a population increases and indicates the reliability of negative tested results.

The Agar plate method evaluated after 48 hours showed a sensitivity of 60 %, a specificity of 96.2%. The negative predictive value was 97.7% and the positive predictive value 47.7%.

The lowest sensitivity was found for the modified Harada Mori technique, assessed 5 to 6 days after preparation, with a sensitivity of 36.4% and negative predictive value of 97.1%. The calculated specificity was 96. 4% and the positive predictive value of 30.7%.

The FPC method and following microscopy showed a sensitivity of 46.7% and specificity of 100%. The negative predictive value was calculated with 97.0% and positive predictive value with 100%. A sensitivity of 92.9% with a specificity of 87.8% was found in the in-house ELISA. The negative predictive value was 99.5% and the positive predictive value 28.9%.

5.2.3 Comparison of reliability of tested methods using the Cohen Kappa interrater reliability method

An interrater reliability test of the results was performed in order to assess the level of agreement between the different methods used for the identification of the prevalence of *S. stercoralis* infections. For the determination of the dimension of the chance for agreement between two different methods the interrater reliability was calculated using the Cohen Kappa statistics. The Cohens Kappa analysis was performed using the PASW statistics package for the analysis of the level of agreements between the five diagnostic methods. [82]. The Cohens Kappa correlation analysis results show a low likelihood or poor level of agreement of the results between two different methods if the Cohen Kappa value (CKV) is smaller than 0.2; a slight level of agreement exists if the CKV ranges between 0.21 and 0.40, a moderate likelihood for an agreement exists if the CKV ranges between 0.41 and 0.60; a substantial or high likelihood of an agreement between two different methods exists if the CKV is in the range of 0.61 and 0.80 and an almost perfect agreement is proven if the CKV is above 0.81.

Literature shows that a Cohen Kappa value in the range of 0.61 and 0.80 are indicating a substantial agreement which indicates that the two methods tested are producing reliable results.

Table 4:   Results of Cohens Kappa interrater analysis of the tested different *S. stercoralis* analysis methods:

NO	COMPARED METHODS	COHEN KAPPA VALUE
1	PCR with FPC and microscopy	0.623
2	PCR with Agar plate method	0.499
3	Agar plate with Harada Mori method	0.471
4	Agar plate method with ELISA	0.431
5	PCR with ELISA	0.394
6	Agar plate method with FPC and microscopy	0.361
7	Harada Mori method with FPC and microscopy	0.360
8	PCR with Harada Mori method	0.301
9	Harada Mori method with ELISA	0.204
10	ELISA with FPC and microscopy	0.164

Source: Own calculations

The result of a Cohen Kappa value of 0.623 was only achieved when comparing the Gold Standard PCR with the FPC method. The comparisons of the PCR and Agar plate, the Agar plate with the Harada Mori and the Agar plate with the ELISA method resulted in moderate Cohen's kappa values of 0.499, 0.471 and 0.431, respectively. The comparisons between PCR and ELISA, Agar Plate with FPC and

microscopy and PCR with Harada Mori showed slight levels of agreements only, since their Cohen's kappa values were ranging between 0.40 and 0.21. Hence, the predictive values of these applied methods for reliably identifying the infection are low. The Cohen kappa values smaller than 0.2 indicate that the comparison of the Harada Mori with Elisa and the ELISA with FPC and microscopy method have a poor agreement and no predictive value at all.[82].

Mc Hugh reported an interrater agreement less than 0.6 shows inadequate agreements and therefore little confidence should be given to methods which produce these results.[83] Therefore it can be concluded that only the FPC and microscopy method compared with the realtime PCR achieved a substantial level of agreement which means that both tests have a moderate interrater reliability or extent of agreement for producing reliable results.

6. Discussion and Recommendations

The risk factors of gaining a soil transmitted helminthic infection was analysed in a meta-analysis by *Strunz et al.* who examined a total number of 94 studies and found in twelve of these studies a scientific relation between sanitation, water supply, shoe-wearing and access to toilet facilities with obtaining a *S. stercoralis* infection. Highly significant correlations were for wearing shoes and having toilet access. However, handwashing after defecation showed no significant relation between obtaining an infection. [84] A study by *Kaminsky et al.* revealed a positive correlation between the infection with *S. stercoralis* and living in a rural area.[85] According to the World Population review 2016 about 80% of the Population in the Republic of Tanzania lives in rural areas. [86] and about 87% of the population in Tanzania have no access tap water and to improved sanitation UNICEF.[87]

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Consequently, the findings of *Strunz et al.* and *Kaminski et al.* show that the Tanzanian population at risk in obtaining *S. stercoralis* infections.

The patients who participated in the study do not correspond to the average living conditions of the Tanzanian population when it comes to location, sanitation and water supply since 90% were coming from the urban area of Mwanza, 92 % declared that they are having access to tap water, 57% of participants stated that they have access to a flush toilets and 41% had at least access to latrines.

The analysis of these investigated risk factors showed no statistically significant correlation for gaining a *S. stercoralis* infection in its p- values at the 95% confidence interval. This can be explained by the rather unilateral study population and the insufficient sample size resulting in too small cases for the different investigated factors.

However, the results of the OR analysis of this study confirm the existing relation for sanitation, here the availability of a latrine or flush toilet and indicate an association for the factor profession, here farmers. These results confirm the findings of *Strunz et al.* and *Kaminski et al.* despite the rather urban biased study population. Consequently, these results indicate a high likelihood that the prevalence of a *S. stercoralis* infection within the rural population of Tanzania is higher than the one identified in the study population due to its urban bias.

Many studies have proven that improvements in of water and sanitation have a positive correlation to the improvement of the general health status, to the reduction of diarrhoea morbidity and reduction of infections with soil transmitted helminths as also shown for the *S. stercoralis* in this study. [63, 84, 87-89]

The five different methods compared in the study indicate that none of the applied method can be established in a low resource setting -such as in the hospital of Mwanza- as a routine procedure in order to detect a *S. stercoralis* infection reliably before starting an ART treatment.

Cultural methods as the Agar plate method and Harada Mori method showed moderate and low sensitivity compared to the PCR analysis. Higher sensitivity, especially concerning the Agar Plate method, could have been reached through consecutive examination of stool samples as described in several studies.[4, 32, 78] Consequently, especially infections with low intensity and parasitic load can be detected.[31] In the present study consecutive stool examinations were not feasible due to logistical limitations since mainly outpatients attending the Care and Treatment Centre (CTC) participated in the study. Compared to the literature results, where the Agar plate method is usually described as a sensitive method with sensitivity levels above 80% for diagnosing an infection, the sensitivity level in this study was rather moderate with 60% only.[29, 76, 81]

Advantages of the Agar Plate method are the high sensitivity compared to the FPC method and a high specificity as well as a high negative predictive value. The Agar plate method requires a special microscope which was not available in the Bugando Hospital for implementation. The equipment had to be brought from Germany and is generally not available in every laboratory in low resource settings. This presents a definite disadvantage of the method. Moreover, the Agar plate method is time consuming as the culture plates need to be evaluated after 24, 48 and 72 hours. In addition, the differentiation between a Hookworm and *S. stercoralis* larvae is rather difficult, as the discrepancy of furrows left in the agar does not always become obvious. Furrows left in the agar are results of bacterial growth along the paths of motile larvae.[81] In the study carried out by *Ines et al.* the correlation between incubation time and positive results of either Hookworm or *S. stercoralis* larvae is described. Short incubation times correlate with the presence of *S. stercoralis* larvae whereas longer incubation time lead to an increasing detection rate of Hookworm larvae. This is caused by the fact that Hookworm eggs need some days for hatching and the development of larvae.[81]

This particular fact could be proven by the PCR analysis in the current study since only samples after 24 hours have been positive with the agar plate method. Whereas samples which seemed positive in the Agar plate method for *S. stercoralis* larvae after 72 hours showed negative results in the PCR analysis. Therefore, it can be concluded that Hookworm larvae were present within these samples and led to a misinterpretation by microscope analysis of the Agar plate as *S. stercoralis* larvae.

One sample which was claimed positive for *S. stercoralis* larvae in the Agar plate method showed a Hookworm egg in the FPC method. This proves the difficulty of diagnosing a *S. stercoralis* infection with the Agar plate culture just by the appearances of furrows. Under certain circumstances the differentiation between Hookworm larvae and *S. stercoralis* larvae is difficult and the interpretation depends on the skills and experience of the examiner. Moreover, two of the false positive Agar plates were contaminated by larvae of flies -as shown in the following picture- which made the identification of furrows more difficult.

Photograph 8: Fly larvae led to contamination of the Agar Plate method
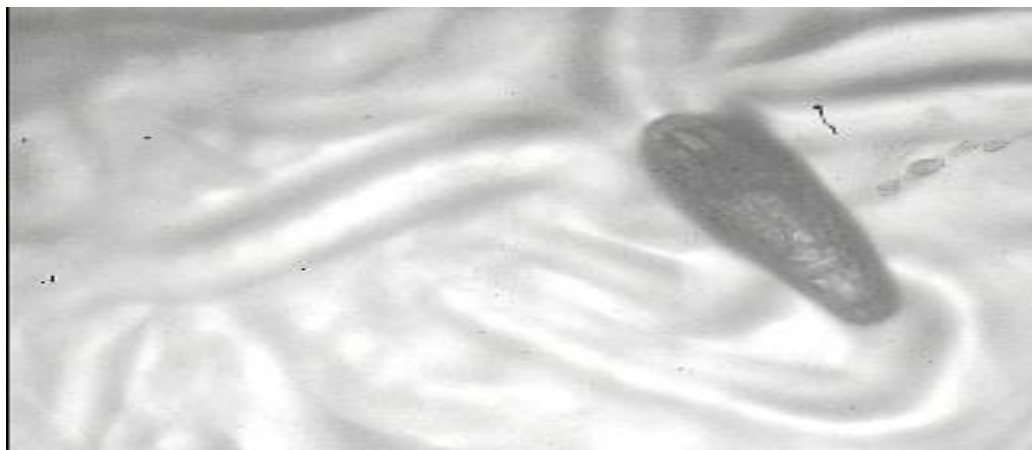
Own Source

The modified Harada Mori technique showed a very low sensitivity of only 36.4 % for the detection of a *S. stercoralis* infection compared to PCR as assumed gold standard. This lack of sensitivity is a result of the difficulty and inadequate precision for the differentiation between Hookworm and *S.*

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stercoralis infection. Thus, in three faecal samples Hookworm eggs could be identified in the microscopy analysis which showed positive results for *S. stercoralis* larvae with the Harada Mori technique. Therefore, the likelihood of false positive infection results with the modified Harada Mori culture technique is high. In addition, the method is time consuming since the analysis of the samples needs to be done after two, three and five to six days. It was experienced that after longer incubation periods faecal particles sedimented on the coverslip making the microscopy analysis difficult or impossible causing the loss of 16 samples. Despite some advantages of the applied modified version the need of a special, inverted microscope and dependence on the skills of the examining lab assistant are clear disadvantages of this method. The identified low sensitivity with the applied method was also observed in respective studies using the classical Harada Mori Technique for the diagnosis of Strongyloidiasis. [51] These findings and disadvantages render even the modified Harada Mori method a relatively unusable method to diagnose a *S. stercoralis* infection.

The FPC method and the subsequent microscopy had a great advantage in order to differentiate between different helminth infections. Therefore, in total three Hookworm eggs could be identified. In 17 samples *Schistosoma mansoni*, was found. *Ascaris lumbricoides*, *Entamoeba coli* and *Jodamoeba bütschlii* cysts were detected each in one sample. The PCR analysis showed that the three samples with evidence of a Hookworm egg were negative within the *S. stercoralis*.
However, the FPC method showed the highest specificity with 100% and positive predictive value with 100 %. As described before the concentration method has similar results as the spontaneous sedimentation technique but better results in comparison to direct examination of fresh specimen. [77]

A sensitivity of only 46.7% as calculated in the present study using the FPC method is even higher compared to studies using the spontaneous sedimentation methods to visualize the *S. stercoralis* larvae. [51, 52]

Disadvantages of the FPC method are the necessity of a special tool kit for performing the faecal concentration as well as its time requirement and its dependency on the microscopy skills of the lab assistant. Consequently, the FPC method does not seem to be a suitable tool for detecting the larvae.

In recent studies diagnosing an infection using molecular methods such as the PCR are increasingly in focus for identifying infections. Highest results in specificity and sensitivity have also been described in cases of chronic infections and immunosuppression.[22, 56, 59, 90] Thus the realtime PCR is assumed as Goldstandard in the present study. In no positive PCR result a Hookworm egg was identified using the FPC method and all results being positive in the FPC method were confirmed by PCR. This internal correlation is also reflected by a high Cohen's kappa value of 0.623 which indicates substantial agreement. In a few cases where larvae were detected in the faecal culture tests, the PCR for Strongyloides remained negative. To confirm that the observed larvae were Hookworm larvae it was tried to establish a realtime PCR with Hookworm specific primers. Unfortunately, the attempt failed due to problems in isolating the Hookworm DNA

The requirement of special laboratory equipment and expensive consumables costing approximately five Euro per sample are disadvantages of the realtime PCR and disallow this method being established as a routine procedure in low resource settings.

The in house ELISA performed in the study showed the highest sensitivity with 92.7% but revealed a lack of specificity with 87.8% through false positive results which is also described in a study by *Ahmad et al.* [62]

Low specificity is probably due to cross reactions with other helminth infections such as filariae, *ascariasis* and acute schistosomiasis.[91]
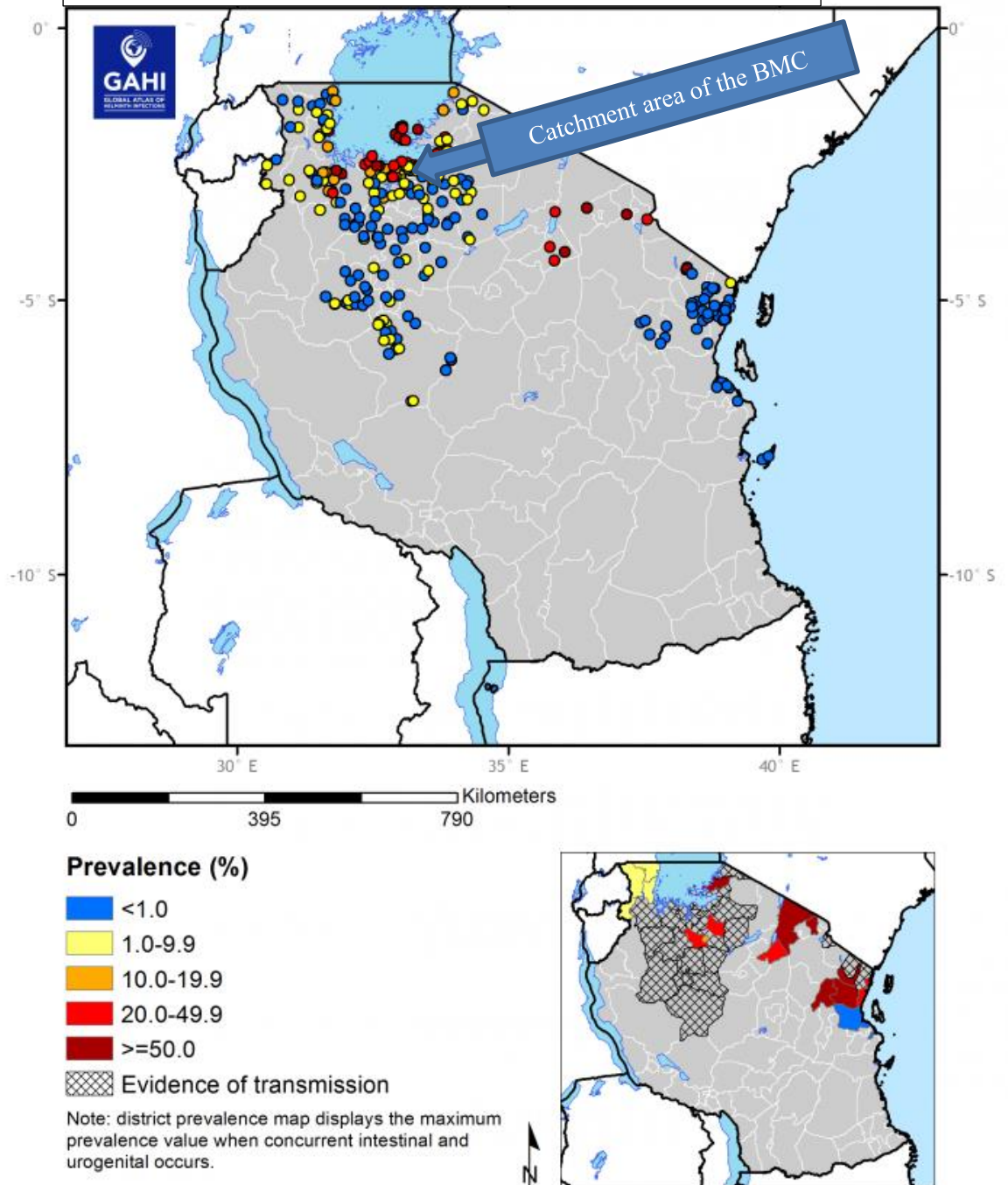
Moreover, the ELISA does not exclusively indicate the presence of a current infection and may be negative in immunocompromised patients.[59, 92]

Gam et al. applied an in-house ELISA using larvae antigens from *S. ratti* and compared it to an ELISA using larvae antigens from *S. stercoralis* which proved cross reactions with sera from patients who had occult filariasis and/or acute schistosomiasis. Moreover, he reported nonspecific reactions when using the in-house ELISA with antigens from *S. ratti* resulting in false positive reactions. These findings indicate that the high amount of false positive results in this study can be a result of the used in house ELISA with the antigens from *S. ratti* larvae.[93] The described cross reactions with acute schistosomiasis could explain the high prevalence results of the in house ELISA used in this study. Schistosomiasis is highly prevalent in Tanzania, especially in the area around Lake Victoria and the catchment area of the Bugando Medical Centre as shown in figure 9, at page 55.

Therefore, the implementation of the used in house ELISA, using larvae antigens from *S. ratti*, as a screening method for *S. stercoralis* infections in settings with high likelihoods of prevalence's of parasitic infections is not recommendable. Furthermore, the implementation of an ELISA in low resource settings is rather difficult due to involved running costs and needed investments for special equipment. However, it should be noted that results of the ELISA highly depend on which larvae antigen is used as well as what kind of pre- incubation steps are done. In a study by *Ahmad et al.* an ELISA, using larvae antigens from *S. stercoralis* is described as a useful screening tool in areas with low prevalence of parasitic infections and may even be used to assess the effectiveness of treatment .[46, 62]

Figure 9: Prevalence and location of *S. mansoni* parasitological surveys ad average district level prevalences in United Republic of Tanzania

Catchment area of the BMC

Prevalence (%)
- <1.0
- 1.0-9.9
- 10.0-19.9
- 20.0-49.9
- >=50.0
- Evidence of transmission

Note: district prevalence map displays the maximum prevalence value when concurrent intestinal and urogenital occurs.

Verification of the results was achieved by calculating the Cohens kappa which is a robust statistic test for interrater reliability. It gets obvious that none of the tested and applied methods shows a high level of interrater agreement.

Mc Hugh describes that an agreement of 0.8 should be reached as an acceptable result and a Cohens kappa less than 0.6 represents an inadequate agreement. In results lower than 0.6 only little confidence should be given. In the present study only the comparison of the FPC method with the realtime PCR showed a result above this threshold (0.62). The calculation of Cohens kappa again summarizes the problems of the different methods to diagnose a *S. stercoralis* infection. Aside from PCR only little confidence can be given to the results. [83]

The aim of this study is to assess the reliability of the available diagnostic methods for *S. stercoralis* infections in HIV positive patients. ART bears the risk of developing an immune reconstitution inflammatory syndrome. [7-9] As described in the study by *French* the possibility of developing an IRIS after receiving ART is particularly high in patients having a Tuberculosis co-infection.[8] According to the WHO TB report 2015 the United Republic of Tanzania belongs to the 22 high burden TB countries of the world. Out of 63151 new TB cases in 2014 35% were HIV positive.[6] This corresponds with the results of this study as 26% of the participating patients stated having a current or past tuberculosis co-infection.

Until now the preferred treatment of severe IRIS is the use of high dose corticosteroid drugs especially in case of a TB IRIS.[8, 71, 72]

As stated in recent studies high dosages of corticosteroid treatment reveal the risk of developing a *S. stercoralis* hyperinfection syndrome and/or disseminated Strongyloidiasis with high mortality rates in case of an unrecognized infection.[2, 13, 34, 39]

Therefore, the importance of ruling out a possible infection with *S. stercoralis* in HIV Aids patients is crucial.[13, 26, 34]

The findings of the present study once more prove the difficulties of diagnosing a *S. stercoralis* infection, especially in laboratories in low resource settings with high prevalences of parasitic infections in the study area. The implemented deworming programs with mass drug administration (MDA) of albendazole / praziquantel aim to reduce the morbidity of soil transmitted helminths but they do not aim to treat or eliminate *S. stercoralis* infections. Furthermore, clinical signs of a disseminated Strongyloidiasis are very unspecific and make the diagnosis very difficult.

The literature analysis and the findings of this study lead to the conclusion that it is high time to consider the provision of a prophylactic treatment within patients who are either HIV positive patients who could develop an IRIS after receiving ART, patients with a HTLV-1 infection and the growing number of patients under iatrogenic immunosuppression for various reasons. For these patients a prophylactic treatment with either ivermectin 200µg/ kg body weight on two consecutive days or albendazole 400 mg twice daily for seven consecutive days should be considered to rule out the risk of a disseminated Strongyloidiasis and/ or hyperinfection syndrome

7. Appendixes

7.1 Ethical Clearance

CUHAS/BMC RESEARCH ETHICAL COMMITTEE (CREC)
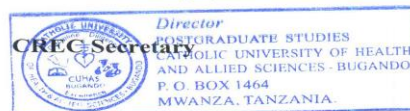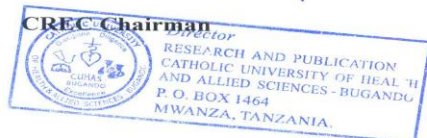
Research Clearance Certificate No. CREC/050/2013

Ethical clearance is hereby granted to the following Principal Investigator/Researcher

<u>PROF. SAMUEL KALLUVYA OF THE DEPARTMENT OF INTERNAL MEDICINE, CATHOLIC UNIVERSITY OF HEALTH AND ALLIED SCIENCES, BUGANDO, MWANZA</u>

On this day of 3RD JUNE 2013 to conduct health research
Involving human subjects titled; <u>PREVALANCE OF STRONGYLOIDES INFECTIONS IN ADULT HIV- POSITIVE PATIENTS AND COMPARISON OF SPECIFICITY AND SENSITIVITY OF FOUR DIFFERENT METHODS TO DETECT THE LARVAE IN NORTHERN TANZANIA.</u>

STRONGPREV-STUDY

Research period is from **JULY 2013 to JUNE 2014.**

CREC Chairman

Director
RESEARCH AND PUBLICATION
CATHOLIC UNIVERSITY OF HEALTH
AND ALLIED SCIENCES - BUGANDO
P. O. BOX 1464
MWANZA, TANZANIA.

CREC Secretary

Director
POSTGRADUATE STUDIES
CATHOLIC UNIVERSITY OF HEALTH
AND ALLIED SCIENCES - BUGANDO
P. O. BOX 1464
MWANZA, TANZANIA.

7.2   Consent Form

Aim of the study:

This study is focussing on an insidious parasitic worm infection which is common in tropical climates like Tanzania. In the state of severe immunosuppression this infection may cause serious illness. Testing for this parasite is not a routine procedure. Therefore, most infections remain undetected.

The study intends to obtain data on the prevalence of Strongyloides infection as a basis for a preventive deworming therapy.

Your voluntary participation is highly appreciated!

What will be done?

You will be asked to answer a few questions related to the risk of acquiring this worm infection. As the infection is transmitted via contaminated soil you will be asked questions concerning living conditions, water supply and sanitation. You will be assigned a participant (study) number. You will be asked to donate a blood sample and a stool sample.

What will be done with the samples?

The blood will be analysed for antibodies to Strongyloides and possibly other worm infections. The stool samples will be analysed by different methods for the presence of Strongyloides infection.

What will not be done?

Your personnel data (name, age, hospital file number, mobile phone number) will only be used to report the results of the investigations to you for eventual treatment. You can refuse recording of personnel data but then a report of results will not be possible

What is the benefit for the participant?

The result of the most sensitive tests for *S. stercoralis*, the culture tests, will be ready one week after delivery of a stool sample. These tests are focussing specifically at *S. stercoralis* – infection and will not give information about other worm infections like ascariasis or schistosomiasis. You can obtain your result from the study nurse on the phone.

What are the risks for the participant?

Blood sampling will be done with sterile safety equipment in the most convenient way for the participant. Blood sampling is a routine procedure in medicine with minor risks only, e.g. local bleeding, swelling or bruises at the site of vein puncture. The risk of an infection by blood sampling is very low. Delivering a stool samples does not have any risks for the participant.

Will the participant be paid?

No. There will be no remuneration for participating in the study.

Will I be offered treatment in case a Strongyloides infection is detected?

If you are staying in the hospital or attending CTC the required deworming tablets will be prescribed.

Who can I contact in case of additional questions or to retrieve my results?

Study Nurse: Sr. Lydia Makenge, CTC clinic, Bugando, mobile no. 071 320 3521

Principal Investigator: Prof. Samuel Kalluvya, Bugando, mobile no. 071 333 0766

I have read the information above. All my questions have been answered and I agree to participate in the study.

Date: Place: Signature: Signature of Witness

7.3   Consent Form ya Idhini

Lengo la utapiti

Utapiti Imu uni kulenga zaidi maambukizi nyemelezi ya vimelea minyoo ambavyo ni kawaida katika hati ya hewa ya Tanzania. Katiku upungupu wa kinga haya maambukizi ni katarishi. Upimaji wa haya magonjwa sio ya utaratibu kwahiyo maambukizi huwa yasiyotambuliwa.

Utapiti huu utaangalia hali ya maammbukizi ya strongyloides ikiwa na lengo la kuzuia maambukizi kwa utumiaji dawa.

Hiari yako katika somo hihi ni wa umuwhimu.

Nihi kitakechopanywa?

Utaulizwa maswali machache kutusu njia hatarishi zakupata magonjwa haya. Maambukizi hupatikana kupitia udongo wenye vimelea hivyo maswali yatahusisha hali ya maisha, upatikanaji wa maji na usapi. Utapatiwa namba ya somo na kuombwa kutoa sampuli ya damu na choo kikubwa

Nini kitakachopanyika na sampuli hizo?

Damu itapanyiwa utapiti kuona umepo wa chembe chembe za kinga shidi ya minyoo hao na wengineo, na choo kikubwa pia kitapanyiwa utapiti kwa njia topauti kuona uwepo wa vimelea hivyo.

Nini hakitapanywa?

Mambo yako binapsi (jina, umi, namba ya pile, namba ya simu) havitatumika katika ripoti isipokuwa pale katika majibu ya vipimo ili uweze pata tiba. Unaweza kataa kutoa mambo yako ya binapsi ila utashindwa kupata majibu.

Ni paida gani utakayopata?

Kupata majibu ya vipimo baada ya wiki moja. Vipimo vinaangalia vimelea vya strongyloides na sio vinginevyo. Unaweza patamajibu kutoka kwa nesi kupitia simu.

Nini ni hatarishi kwako?

Vipimo vya damu vitachukuliwa kwa usapi kwa ushirikiano wa mgonjwa. Unaweza toka damu kidogo, kuvimba kwenya sehemu utakayochomwa sindano. Kupata maambukizi mengineyo ni kidogo sana. Utoaji wa choo kikubwa hauna hatari yoyote.

Je utalipwa?

Hapana, hapatakuwe na malipo ya ania yoyote.

Je utapewa tiba?

Iwapo umekutwa na maambukizi ya vimelea hivyo tiba itapewa kwako.

Nani wakumunlizia iwapo uina maswali au kupata majibu?

Nesi, Sr. Lydia Makenge wa CTC Bugando, namba ya simu 0713203521

Mtapiti mkuu, Propesa Samuel Kalluvya, Bugando, namba ya simu 0713330766

Nimesoma taanipa zote juu, maswali yangu yote yamejibiwa na ninakubali kuhusishwa katika somo/ stadi hii.

Tarehe :   Sehemu:

Shihi: Sahihi ya shahid

7.4   Patient questionnaire:

1. Namba ya simu:

Mobile number

2. File Nr:

3. Study Nr:

4. Ni jimbo/ wilaya gani unaishi?

In which Ward/ District do you live?

5. HIV positive and admitted: ☐

Kulazwa kwa ajili :   Shida ya pumzi: ☐

Admission because of   *Respiratory problems*

                                                              Homa: ☐

                                                              Fever

                                                              Matatizo ya matumbo: ☐

                                                              Abdominal problems

                                                              Nyingine: ☐

                                                              Other _____

6. HIV- positive at CTC: ☐

7. HIV-negative: ☐

8. Umri: (*Age)*   _____

9. Mwanamke (*Female)***:** ☐   **Mwanaume** *(Male)***:** ☐

10. Kazi: Mvuvi: ☐   Mkulima: ☐   Nyingine: ☐

Profession   Fishermen   *Farmer*   *Other*

11. Makazi: Mjini: ☐   Vijijini: ☐   Kitongoji: ☐

Living condition: Urban   *Rural*   *Suburb*

12. Maji unayotumia: Bomba: ☐   Kisima: ☐   Chemchemi: ☐

Access to water   *Tap*   *Well*   *Spring*

Mto/ Ziwa: ☐

River/Lake

13. Mna choo nyumbani: Ndio: ☐   Hapana: ☐

Sanitary supply; Toilet at home   *Yes*   *No*

Kama Hapana unatumia nini: Kichaka: ☐ Choo cha shimo: ☐

When No; using the: *Bush*   *Latrine*

Choo cha: ☐

Flush toilet

15. Unavaa viatu: Mara chache: ☐ Sivai: ☐   Mara zote: ☐

Wearing shoes   *Often*   *Never*   *Always*

16. Historia ya magonjwa: Kifua kikuu: Ndio: ☐   Hapana: ☐ Sijui: ☐

History of illness: *Tuberculosis Yes*   *No*   *Don´t know*

17. Kisukari: Ndio: ☐ Hapana: ☐   Sijui: ☐

Diagnosed Diabetes Mellitus   *Yes*   *No*   *Don´t know*

18. Kubanwa kifua: Ndio: ☐   Hapana: ☐   Sijui: ☐

Diagnosed Allergic Asthma   *Yes*   *No*   *Don´t know*

19. Umepata dawa za minyoo ndani ya miezi 12?

Deworming/Anthelmintic therapy within the last 12 months?

Ndio: ☐   Hapana: ☐   Sijui: ☐

Yes   *No*   *Don´t know*

Kama Ndio: Albendazol: ☐   Praziquantel: ☐ Sijui: ☐

Dozi:_____   Dozi:_____   *Don´t know*

7.5   File for Study Nurses/ Study assistant:

Information taken from patient files:

1. Mobile number: _____

2. File Nr: _____

3. Study Nr: _____

4. Age: _____

5. HIV and admitted: ☐

6. Admission because of: Respiratory problems: ☐

Fever: ☐

Abdominal problems: ☐

Other: ☐

_____

7. HIV in CTC: ☐

8. CD 4 count: last:_____ lowest:_____

9. Antiretroviral therapy: Yes: ☐   No: ☐

10. Treatment with current Corticosteroids (Prednisolon, Dexthamethason):   Yes: ☐   No: ☐

   When Yes: Dose:_____

11. Treatment with other immunosuppressing drugs:

Yes: ☐   No: ☐

When Yes: Dose: _____

12. Deworming/ Anthelmintic therapy within the last 12 months:

Yes: ☐   No: ☐   Unknown: ☐

When yes: Albendazol: ☐; Dose:_____;

Praziquantel: ☐; Dose:_____

Unknown: ☐

13. History of additional chronic illness:

Tuberculosis: Yes: ☐ No: ☐

Pneumocystis pneumonia: Yes: ☐ No: ☐

Cryptococcus meningitis: Yes: ☐ No: ☐

Diagnosed: Diabetes mellitus: Yes: ☐ No: ☐

Allergic asthma: Yes: ☐ No: ☐

8. Literature review

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