Morbidity Assessment and Diagnosis of Schistosomiasis Haematobium in Female Reproductive Organs by the Detection of Schistosome Circulating Antigens

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To my loving and supporting family and friends..
God bless them all.

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Preface

Among human parasitic diseases, schistosomiasis or bilharziasis has a significant importance on socio-economics and public health in tropical and subtropical areas. There are five *Schistosoma* species that are capable of infecting humans; they differ in a number of important ways, including the final location in the host, number of ova produced by a pair of worms, size and shape of the egg, and the morbidity they induce in the host. These differences are expressed as different clinical and pathological features among infected patients. Morbidity in human reproductive organs due to schistosomiasis, especially *Schistosoma haematobium* infection, is a known but neglected manifestation. Recently, many specialists in tropical diseases started to draw attention to genital schistosomiasis. Schistosomiaisis of the genital tract may seriously affect the reproductivity of the infected individual and may cause irreversible pathological consequences. In the case of female genital schistosomiasis (FGS), there are several ways in which schistosomiasis may influence fertility. Appropriate diagnostic tools for FGS are not at hand and a therapeutic rationale does not exist. Unfortunately, the pathological patterns and immunology of genital lesions are not well known. How afflicted women perceive their symptoms and their role in connection to transmission and control of schistosomiasis have never been studied.

In this thesis, **chapter one** introduces genital schistomiasis with special emphasis on female genital schistosomiasis (FGS). **Chapter two** reviews human schistosomiasis and different morbidity patterns, including genital schistosomiasis, associated with the infection. Moreover, different methods used for the diagnosis of schistosomiasis and general perspectives on treatment and control are presented in this chapter. **Chapters three and four** describes a case control study. The methodologies followed to assess morbidity in genital organs due to *S. haematobium* infection among females at the childbearing age; and the use of monoclonal antibody based immunoassays for the diagnosis of female genital schistosomiasis are described in the prior chapter and outcomes are detailed in the later one. **Chapter five** discusses the current findings and its

contribution to research on schistosomiasis as well as social and gender considerations.

Some of the findings in this thesis may contribute to the efforts that have been made by other researchers in order to assess morbidity due to schistosomiasis haematobium in female genital organs; and may thus serve as a guiding lead to better understanding of this important manifestation of schistosomiasis. Different tools were used to diagnose female genital schistosomiasis; testing circulating antigens in cervico-vaginal secretions could be an interesting approach and may provide valuable information on host-parasite relationship.

Abstract

Schistosomiasis haematobium is an important public health problem in Africa and the Middle East and the infection causes considerable morbidity in a high proportion of cases (Warren *et al.,* 1979). The frequency of genital schistosomiasis was encountered in areas where infection with *Schistosoma haematobium* prevails. It also occurs with *S. mansoni*, *S. mattheei* and more rarely *S. intercalatum* (Smith and Christie, 1986). Schistosomiasis haematobium related morbidity in females' reproductive organs in the Midwestern Sudan was investigated. In total, 118 females at the childbearing age (15-50 years old) were enrolled in the study after being informed consent. The application of immunoassay techniques to detect schistosome antigens in genital secretions was evaluated. Females at the childbearing age having gynecological complaints were submitted to selection criteria for a case control study. The study investigated cases infected with *S. haematobium* and negative controls from Rahad area-Midwestern Sudan that is endemic for schistosomiasis haematobium. A group of negative controls from a non-endemic area (Khartoum area-central Sudan) were also included. Sample collection as well as parasitological, clinical, ultrasonography and gynecological examination were conducted in the hospital following the selection of cases and controls. Treatment was given to patients as directed by the physician. Monoclonal antibody based enzyme-linked immunosorbent assay (ELISA) was used to determine the levels of schistosome circulating cathodic antigen (CCA), circulating anodic antigen (CAA) as well as soluble egg antigen (SEA) in urine, serum and vaginal wash samples. Thirty-four (28.8%) were cases from Rahad area and 84 (71.2%) were controls, of whom 45 (38.1%) were from Rahad area and 39 (33.1%) were from Khartoum area, representing an

approximate 1:2 cases to controls ratio. Significant differences in morbidity patterns were found between cases and controls. Antigen levels varied between study groups and significant differences in vaginal wash CCA, serum CAA as well as urine SEA levels were found between cases and controls; and coincided with pathological findings reported by ultrasonography and gynecological investigations. Significant correlations of morbidity with antigen levels were also found. The use of circulating antigens determination in cervico-vaginal secretions, especially CCA, has the potential to be used as a diagnostic method for FGS. The measurement of SEA in vaginal wash sample can also be used as a confirmatory test.

ملخص الدراسة

مرض البلهارسيا البولية تنعكس أهميته على الصحة العامة في أفريقيا و الشرق الأوسط، كما أن أثاره المرضية تصيب نسبة كبيرة من الحالات. تم تسجيل الكثير من حالات البلهارسيا التناسلية في مناطق انتشار طفيل المنشقة الدموية، و من الممكن العثور على مثل هذه الحالات في مناطق انتشار طفيل المنشقة المانسونية، و المنشقة الماتثية، و بصورة نادرة في مناطق انتشار طفيل منشقة انتركالاتم. تهدف الدراسة الحالية لاستكشاف المرضية المتسببة من البلهارسيا البولية في الأعضاء التناسلية للنساء بغرب و وسط السودان، و لتقييم أداء التقنيات المناعية في الكشف عن مستويات مستضدات طفيل البلهارسيا في الإفرازات التناسلية. تم إخضاع عدد من النساء في مرحلة الخصوبة (15-50 سنة) و اللائي يعانين من بعض المشاكل التناسلية لدراسة تعتمد على مقارنة الحالات المصابة بأشخاص غير مصابين. فحصت الحالات المصابة بالبلهارسيا البولية و قورنت بأشخاص من منطقة الرهد التي تنتشر فيها البلهارسيا البولية كما تمت المقارنة بأشخاص من منطقة ليس بها انتشار للبلهارسيا البولية (من ولاية الخرطوم). تم جمع العينات بالإضافة للفحص الطبي و بالمستشفى كما تم فحص الجميع بالموجات الصوتية بواسطة طبيب أخصائي و عولجت جميع الحالات بناء على توصية الطبيب المختص. استعملت تقنية المناعة الامتصاصية ELISA لتحديد مستويات مستضد البلهارسيا الأنودي CAA، و مستضد البلهارسيا الكاثودي CCA، ومستضد بيض البلهارسيا القابل للذوبان SEA في عينات من البول و الدم و السائل المهبلي. العدد الكلي من النساء اللاتي أخضعن للدراسة بلغ مائة و ثمانية عشر سيدة بعد التأكد من أخذ موافقتهن. أربعة و ثلاثين سيدة (28.8%) تم اختيارهن كحالات مصابة بالبلهارسيا و 45 (38.1%) حالة للمقارنة من منطقة الرهد بالإضافة إلى 39 (33%) حالة للمقارنة من منطقة الخرطوم ممثلين بذلك نسبة 2:1 من الحالات المقارنة بالنسبة إلى الحالات المصابة. كان هناك اختلاف معنوي في الحالات المرضية التي وجدت بين المجموعات المختارة كما كان هناك تفاوت في مستويات مستضدات البلهارسيا. رصد تباين معنوي في مستويات ال CAA في الدم، و ال CCA في السائل المهبلي و ال SEA في البول بين الحالات المصابة بالبلهارسيا و الحالات المقارنة. تم اختبار العلاقة بين الحالات المرضية و مستويات مستضدات البلهارسيا كما تم تقييم و مناقشة إمكانية استخدام تقنية المناعة الامتصاصية ELISA لتحديد مستوى مستضدات البلهارسيا في السائل المهبلي كوسيلة لتشخيص البلهارسيا التناسلية لدى النساء.

1. CHAPTER ONE: INTRODUCTION

1.1. Genital schistosomiasis

Schistosomiasis is considered to be one of the major parasitic diseases in the world. Schistosomiasis haematobium is an important public health problem in Africa and the Middle East and the infection causes considerable morbidity in a high proportion of cases (Warren *et al.,* 1979). In contrast to other parasite species causing human schistosomiasis, the oviposition of *S. haematobium* is not uniform and pathological lesions have been described in many pelvic organs (Chen and Mott, 1989; Feldmeier *et al.,* 1994). This explains why eggs are not only found in the urine but also in the stool and in genital organs. Indeed, eggs of *S. haematobium* are so frequently encountered in rectal and genital organs that the customary designation, ectopic localization, seems unwarranted (Chen and Mott, 1989). The frequency of genital schistosomiasis was encountered in areas where infection with *S. haematobium* prevails. It also occurs with *S. mattheei* and more rarely *S. intercalatum* (Smith and Christie, 1986). To which extent *S. mansoni, S. japonicum* and *S. intercalatum* cause genital lesions is still a matter of debate (Feldmeier *et al.,* 1995). Less well known is the gender difference in disease manifestations in women and men and the perception and meanings given to these manifestations by both men and women (Ferguson, 1990).

1.2. Female genital schistosomiasis (FGS)

Female genital schistosomiasis (FGS) is defined as the presence of ova and/or a characteristic pathology due to schistosomiasis in reproductive organs of females (Poggensee and Feldmeier, 2001). As a result of extensive gynaecological and pathological studies by Gelfand and Ross (1953), they came out with the hypothesis that if a female has urinary bilharziaisis, she will probably have ova of *S. haematobium* in some portion of the genital tract. *S. haematobium* worms do not stay at a certain anatomic site their whole life span, but tend to explore the adjacent vascular beds, making multiple organ lesions due to sequestered eggs (Smith and Christie, 1986). Once the adult worms pass the anorectal plexus after they leave the portal vein in the liver, they gain easy access to the perivesical plexus as well as to the plexus utero-vaginalis. The abundant anastomoses between the different venous plexus of the small pelvis, the veins of which are almost without valves and allow blood to flow in either direction, a case that offers a network for routes for the migration of worms to any genital organ. Eggs deposited in the wrong site find them

selves in an anatomical impasse and induce the formation of a peri-oval granuloma, the accumulation of which eventually leads to destruction of soft tissue, fibrosis and formation of scars. The ensuing pathology is presumably independent of the way eggs have reached the genital tissue, i. e. through direct oviposition at the anatomic site or passive transport with the blood flow from a topographically distant location (Poggensee and Feldmeier, 2001). Adaptive changes in pelvic vascularisation and changes in the direction of blood flow during pregnancy further increase the chances of adult worms to settle in genital organs during the reproductive age (Magdi, 1974; Moore and Smith, 1989). The venous vasulature of the female pelvis as described by Poggensee *et al.,* (1999) is illustrated below in figure (3).

The various types of lesions and the broad spectrum of clinical manifestations are assumable expressions of a complex delayed type of hypersensitivity response to sequestered viable, dying or dead ova (Smith and Christie, 1986). Spontaneous healing after interruption of re-exposure may occur, but cases have been reported where uro-genital lesions led to complications many years after exposure (Richter, 2000). According to postmortem and histopathological studies, the frequency of FGS in the upper reproductive tract (URT) ranged from 2% to 83% (Feldmeier *et al.,* 1995). In the lower reproductive tract (LRT), the prevalence was 33%–75% (Renaud *et al.,* 1989; Kjetland *et al.,* 1996; Leutscher *et al.,* 1998). Field studies from Malawi, Tanzania and Madagscar had shown high prevalence of genital lesions caused by *S. haematobium* (Kjetland *et al.,* 1996; Leutscher *et al.,* 1998; Poggensee *et al.,* 2000).

Appropriate diagnostic tools for FGS are not at hand yet and a therapeutic rationale does not exist; the pathophysiology and immunology of genital lesions are imperfectly understood; the natural history of the disease is not well known and it is still a matter of dispute whether genital schistosomiasis promote the development of malignant tumors; how diseased women perceive their symptoms and the role these perceptions play in connection to transmission and control of schistosomiasis has not been studied (Feldmeier *et al.,* 1995).

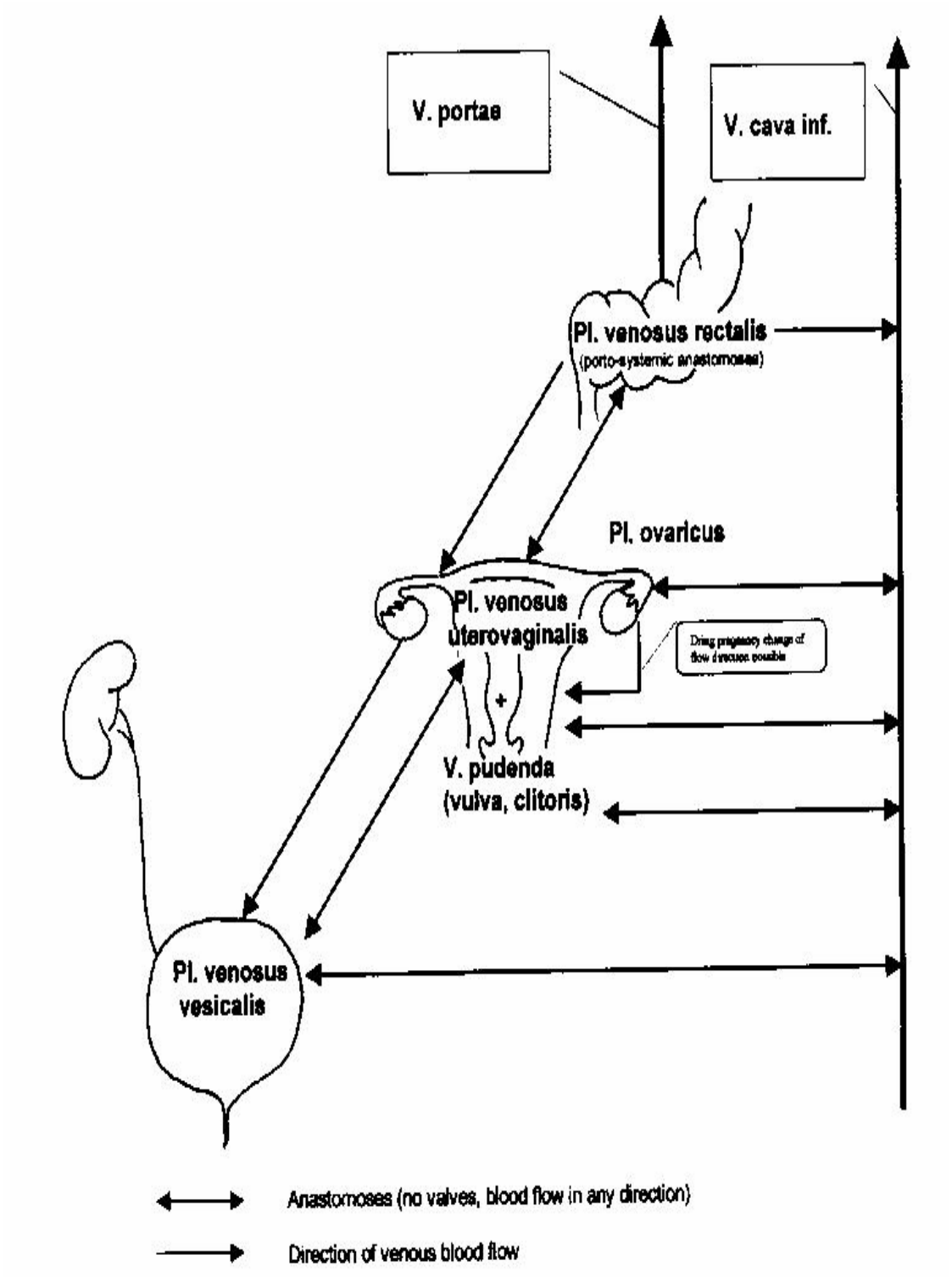


Figure (1): Female pelvis venous vasculature (from Poggensee *et al.,* 1999; also see Poggensee and Feldmeier, 2001).

In the year (2001), Poggensee and Feldmeier summarized the parasitological, clinical and epidemiological characteristics of female genital schistosomiasis and its presumptive interactions with other infectious diseases. Based on clinical findings and available pathophysiological and immunological data, it is conceivable that FGS of the cervix and vagina not only faciltates the infection with agents of sexually transmitted diseases, but presumably also alters the natural history of such infections. Two infectious agents are of concern: the human immunodeficiency virus (HIV) and the onchogenic human papilloma vairus (HPV) (Poggensee and Feldmeier, 2001).

1.3. Diagnosis and morbidity assessment

A potential area of application for a morbidity marker is genital schistosomiasis (Vennervald *et al.,* 2000). The diagnosis of schistosomiasis of the reproductive tract in females is usually made through the incidental observation of schistosome eggs in histological sections made from biopsies routinely taken during gynecological examination or laparoscopy (Bland and Gelfand, 1970). The random nature of diagnosis parallels the fact that the value of most diagnostic procedures is unidentified (Poggensee and Feldmeier, 2001). Four approaches were categorized for the diagnosis of the female lower reproductive tract schistosomiasis: symptoms, macroscopically observable alteration of the genital epithelium, demonstration of eggs in tissue and immunological disease markers presumably released during the development of egg related lesions (Poggensee and Feldmeier, 2001).

1.4. FGS traditional diagnostic approaches

It would be of practical importance to find indicators of FGS by medical history and self reported symptoms. Questions are related to previous and present urinary symptoms, gynecological complaints, pregnancies, deliveries, contraception, menstruation, sexual history, sexually transmitted diseases and previous medical treatment. The risk of being secondary infertile, having menorrhagia, history of genital ulcer and postcoital bleeding were higher among females with genital schistomiasis (Kjetland *et al.,* 1996). Alteration of the genital epithelium could be macroscopically observed using a colposcope. Noting that most probably females in schistosomiasis endemic areas do not regularly subject them selves to gynecological examination, medical history should be taken in a restful atmosphere in order to prepare patient for examination. Gynecological examination is performed with

external inspection followed by internal inspection using a speculum and a colposcope. The risk of bloody cervical discharge, tumors (over all), papillomatous tumors in cervix and vagina and erosions/ulcers in perineum were found to be higher among cases with genital schistosomiasis (Kjetland *et al.,* 1996). The same authors detected *S. haematobium* ova in epithelial material from cervix and suspected lesions and concluded that smears added little to the diagnosis of FGS.

The diagnosis of genital infection in women is relying on biopsies (Vnnervald *et al.,* 2000). Microscopic examination of wet crushed biopsies detected ova in a frequency similar to that of histological sectioning when comparing divided pieces of cervical, vaginal and vulval tissues. The quantitative crushed biopsy technique (QCBT) allowed the diagnosis of genital schistosomiasis to be performed at the bedside and would prevent unnecessary surgery or clinical suspicion of malignancy in situations where histological service is non-existent or unacceptably delayed. However, histological examination is necessary in order to diagnose concomitant pathology such as malignancy and human papilloma virus (HPV) infection (Kjetland *et al.,* 1996).

1.4.1. Ultrasonography

Ultrasonography (US) is suitable for diagnosing schistosomiasis-related organic pathology and is particularly useful to assess its evolution after therapy and/or interruption of exposure to the *Schistosoma* parasites. Intensity and duration of exposure, different parasite strains, patient's age and genetic background all influence the evolution of pathology (Richter, 2000). Although there is indication for varying morbidity patterns in different African foci, it was shown that ultrasonography is useful in the detection of morbidity induced by schistosomiasis on an individual basis and on the community level. An ultrasonographic urinary bladder morbidity score was developed and tested by Medhat *et al.* (1998), and morbidity related to *S. haematobium* infection was assessed in a study made by Serieye *et al.* (1996). Ultrasonographic reports showed urinary bladder masses, wall thickening, calcifications, periappendical and perihepatic adhesions as well as adnexal adhesions/schistosomal salpingitis when using transabdominal ultrasonography, transvaginal ultrasonography ,computed tomography and cystoscopy that was confirmed by laparoscopy and laparotomy (Scully *et al.,* 1994).

1.4.2. Immunodiagnostic assays

1.4.2.1. Host-related immunological disease markers

Eosinophils are found in large numbers in the schistosome egg granulomas (Vennervald *et al.,* 2000). The median eosinophil cationic protein (ECP) concentration in extracts from vaginal lavage were 6 times higher in women with genital shistosomiasis than in women with urinary schistosomiasis but no egg related genital lesions (Poggensee *et al.,* 1996). Immunoglobulin A (IgA), IgG4, IgE, total IgE and neopterin against SEA and adult worm antigen (AWA) were also assessed in vaginal lavage of *S. haematobium* infected patients before and after treatment with parziquantel (Richter *et al.,* 1996).

1.4.2.2. Detection of schistosome circulating antigens

Living parasites excrete and secrete a number of different antigens into the circulation of the host. The detection of these antigens potentially offers a large number of diagnostic, epidemiological and research applications (Van lieshout, 1996). Both schistosome circulating anodic and cathodic antigens (CAA and CCA) were demonstrated in the host's serum, urine, milk (CCA only), kindney, liver and spleen (Deelder *et al.,* 1976; Berggren *et al.,* 1967; Carlier *et al.,* 1975; Santoro *et al.,* 1977; Deelder *et al.,* 1980; Sobh *et al.,* 1987). CAA and CCA were also measured in vaginal lavage before and after treatment (Richter *et al.,* 1996).

As schistosomes can not be directly counted due to their fixed location in the vascular system, antigen levels may thus provide the much needed indirect measure of worm burden to answer important standing questions on the dynamics of schistosome infections, immunity, morbidity and control (Polman, 2000). Initially human studies were mainly focused on the detection of CAA in serum for the diagnosis of schistosomiasis mansoni. However, in later studies CAA and CCA were demonstrated in either serum or urine and in individuals infected with other *Schistosoma* species, with the exception of *S. mekongi*, which has not been examined so far (Van Lieshout, 1996). In the recent years, soluble egg antigen (SEA) has been demonstrated in both serum and urine of *Schistosoma* infected individuals. Unlike the detection of adult worm antigens (CAA and CCA) that provide information on the worm burden, the detection of SEA could provide more information on the egg burden and might thus be a good parameter for the assessment of pathology (Kahama, 1998).

1.5. Aim of the study

The prime aim of the study was to investigate schistosomiasis haematobium related morbidity in the female reproductive organs among females at the childbearing age from the Midwestern Sudan. Application of immunoassay techniques to detect schistosome circulating antigens in genital secretions was a major interest to test and evaluate diagnostic tools for genital schistosomiasis in females.

1.6. Specific objectives:

- To assess morbidity due to *S. haematobium* infection in female reproductive organs using indirect markers (clinical examination, gynecological examination and ultrasonography).
- To measure schistosome-circulating antigen levels in urine serum as well as cervico-vaginal secretions and to test correlation of antigen levels with parasitological and clinical data.

2. CHAPTER TWO: LITRATURE REVIEW

2.1. Human schistosomiasis

Among human parasitic diseases, schistosomiasis (sometimes called bilharziasis) ranks second behind malaria in terms of socio-economic and public health importance in tropical and subtropical areas. The disease is endemic in 74 developing countries, infecting more than 200 million people in rural agricultural and peri-urban areas. Of these, 20 million suffer severe consequences from the disease and 120 million are symptomatic and an estimated 500-600 million people worldwide are at risk from the disease. An estimated 80% of all cases, and all of the most severely affected, is now concentrated in Africa (WHO/OMS, 1998; Chitsulo *et al.,* 2000; WHO/OMS, 2001). Most endemic countries are among the least developed whose health systems face difficulties to provide basic care at the primary health level (Chitsulo *et al.,* 2000). In many areas, schistosomiasis infects a large proportion of under-14 children. Direct mortality is relatively low, but the disease burden is high in terms of chronic pathology and disability (WHO/OMS, 2001). Disease burden accounts for 1,760,000 DALYs and 15,000 Deaths (WHO, 2001; WHO/TDR, 2003a).

The major forms of human schistosomiasis are caused by five species of water-borne flatworm, or blood flukes, called schistosomes. Urinary schistosomiasis, caused by *Schistosoma haematobium*, is endemic in 54 countries in Africa and the Eastern Mediterranean (WHO/OMS, 1998). Intestinal schistosomiasis, caused by *S. mansoni,* occurs in 53 countries in Africa, the Eastern Mediterranean, the Caribbean and South America. Oriental or Asiatic intestinal schistosomiasis, caused

by the *S. japonicum* group of parasites (including *S. mekongi* in the Mekong river basin), is endemic in seven countries in South-East Asia and in the Western Pacific region. Another form of intestinal schistosomiasis, caused by *S. intercalatum,* has been reported from 10 central African countries. The most severely affected countries in Africa are Angola, Central African Republic, Chad, Egypt, Ghana, Madagascar, Malawi, Mozambique, Nigeria, Senegal, Sudan, the United Republic of Tanzania, Zambia, Mali, Uganda and Zimbabwe (Iarotski and Davis, 1981; Taylor and Makura, 1985; Kabatereine *et al.,* 1992; Ndhlovu *et al.,* 1992; Bukenya and Nsungwa, 1994; Kabatereine *et al.,* 1996; Traore *et al.,* 1998; Chitsulo *et al.,* 2000).

S. haematobium is mainly transmitted by *Bulinus* snails, *S. mansoni* by *Biomphalari*a, and *S. japonicum* by amphibious *Oncomelania* snails (WHO/TDR, 2003b). The life cycle of schistosome species infecting human is illustrated below in figure (1) as cited from the CDC/DPDx, (2002). Pathology of *S. mansoni* and *S. japonicum* schistosomiasis includes: Katayama fever, hepatic perisinusoidal egg granulomas, Symmers' pipe stem periportal fibrosis, portal hypertension, and occasional embolic egg granulomas in brain or spinal cord. Pathology of *S. haematobium* schistosomiasis includes: hematuria, scarring, calcification, squamous cell carcinoma, and occasional embolic egg granulomas in brain or spinal cord (CDC/DPDx, 2002).

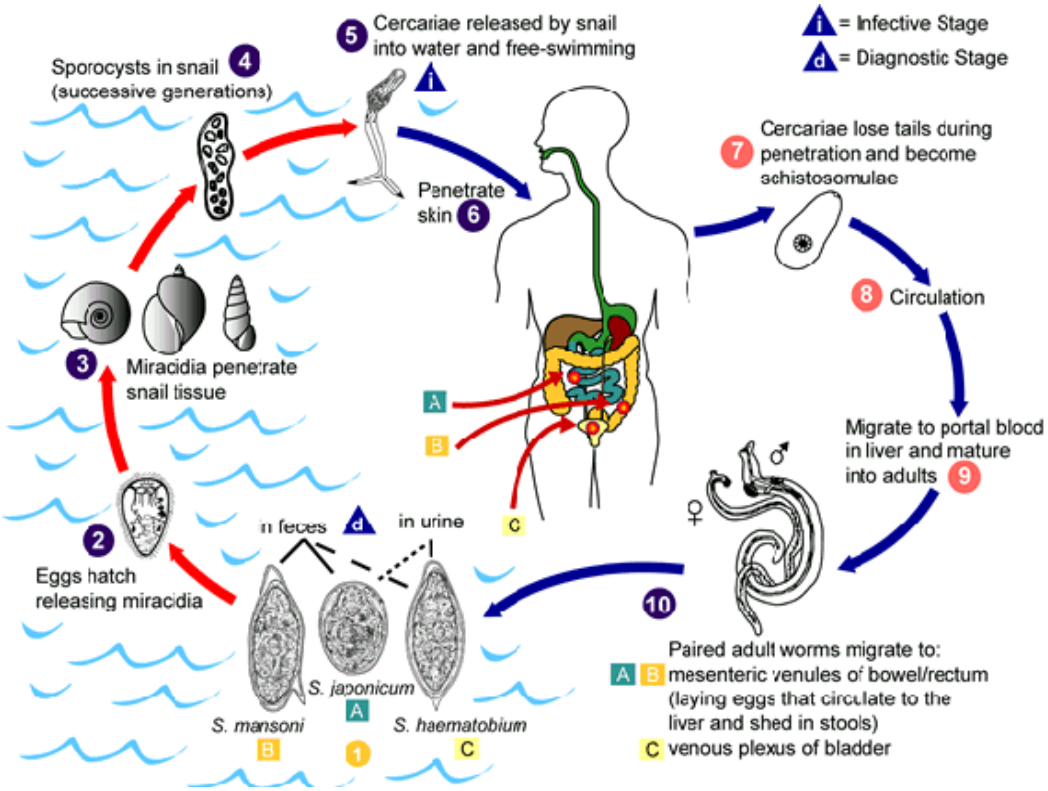


Figure (2): The life cycle of schistosome species infecting human (cited from CDC/DPDx http://www.dpd.cdc.gov/dpdx/HTML/Schistosomiasis.htm).

Basically, schistosome species differ in a number of important ways, including the length of time between penetration and oviposition, final location in the host, number of ova produced by a pair of worms that have morphological differences between species, size and shape of the egg, and the inflammatory reaction they induce in the host (Von Lichtenberg *et al.,* 1973; Warren *et al.,* 1975). These differences probably account for the different clinical and pathologic expressions among infected patients (Nash *et al.,* 1982).

2.2. Morbidity due to schistosomiasis

The major disease manifestations occur in a relatively small percentage of the infected population, usually after years of persistent exposure and infection (Cook *et al.,* 1974; Arap Siongok *et al.,* 1976; Lehman *et al.,* 1976). Although the disease is

widely distributed in a considerable number of countries, morbidity patterns due to schistosome infections and appropriate morbidity markers still need extensive investigations. According to Cheever (1968), the major factor determining morbidity and mortality is the intensity of infection, which is crudely reflected by the number of eggs excreted. The evolution of schistosome infections unfolds over decades. To some extent the symptoms, signs, and prognosis depends on the stage of the disease. The progression of infection from acute to chronic is associated with pathologic changes and modulation of humoral and cellular responses of the host to different stages of the parasite (Nash *et al.,* 1982). There might also be an age dependent factors influencing human host susceptibility to infection (Satti, 1993; Fulford *et al.,* 1998; Ahmed, 1999).

The basic pathology associated with *Schistosoma* infections include the allergic manifestation of the early clinical picture, formation of granuloma, cellular reaction, fibrosis, local complication determined by fibrotic extensions that cause scaring, anemia, hypersplenism where there is splenomegaly following and portal hypertension (Adams and Maegraith, 1984).

2.2.1. Intestinal schistosomiasis

In schistosomiasis from *S. mansoni*, after acute manifestation of initial infection, symptoms subside for a long period despite an ongoing infection (Cook *et al.,* 1974; Arap Siongok *et al.,* 1976; Lehman *et al.,* 1976). Some infected persons continue to develop enlarged livers, which after 10 to 15 years may be associated with splenomegaly (Kloetzel, 1962; Prata and Pina, 1968; Lehman *et al.,* 1976), most likely due to hyperplasia and to portal hypertension caused by liver fibrosis (Prata and Pina, 1968). Increased portal pressure is due to presinusoidal blockage (Coutinho, 1968). Almost all mortality caused by *S. mansoni* is secondary to Symmers' periportal fibrosis of the liver that can now be diagnosed by ultrasonography (Homeida *et al.,* 1988a). Patients have repeated bouts of bleeding due to esophageal varices, and exsanguination is the major cause of death, which most commonly occurs after 3 or 4 decades (Rodriguez *et al.,* 1955; Cheever and Andrade, 1967).

2.2.2. Urinary schistosomiasis

In urinary schistosomiasis due to *S. haematobiu*m, damage to the urinary tract is revealed by blood in the urine. Urination becomes painful and is accompanied by progressive damage to the bladder, ureters and then the kidneys (WHO/TDR, 2003b). In endemic populations, the exudative inflammatory responses that are manifested as mass lesions in the bladder and ureter give way to the irreversible and chronic fibrosing lesions that are usually evident by the third decade (Lehman *et al.,* 1973; McCully *et al.,* 1976). The major symptoms of chronic infection continue to be dysuria and haematuria (Pugh *et al.,* 1980). Obstructive uropathy is usually asymptomatic until well advanced (Nash *et al.,* 1982). Those patients developing renal failure and death secondary to hydronephrosis are few in comparison to the entire infected population (Forsyth, 1969). Squamous cancer of the bladder is a complication of chronic infection (Cheever, 1978), as are disabling, non-healing ulcers of the bladder (Nash *et al.,* 1982).

2.2.3. Genital schistosomiasis

Morbidity in human reproductive organs has been reported as one of the manifestations associated with schistosomiasis, especially *S. haematobium* infection (Manson-Bahr and Bell, 1987). Post mortem studies and histological sectioning of surgical material, especially from the southern part of Africa, have underlined the considerable degree of morbidity associated with schistosome egg granulomas in the female reproductive organs (Gelfand and Ross 1953; Berry 1966; Bland and Gelfand 1970). Recently, high levels of eosinophil cationic protein (ECP), that is associated with the formation of egg granuloma, and *S. haematobium* eggs have been demonstrated in semen from men with genital schistosomiasis haematobium infection (Leutscher *et al.,* 2000).

Schistosomiaisis of the genital tract may seriously affect the reproductivity of the infected individual and may cause irreversible pathological consequences. In female genital schistosomiasis, there are several ways in which schistosomiasis may influence fertility (Helling-Giese *et al.,* 1996a). More over, Feldmeier *et al* (1994) discussed genital schistosomiasis as a candidate facilitator

for the transmission of sexual transmitted diseases (STDs) including human immunodifficiency virus (HIV) infection. The theory of cervical cancer risk related to schistosome infection has been discussed by Kjetland *et al.* (1996).

2.2.4. Schistosomiasis and cancer

Mostafa *et al.,* (1999), extensively discussed the relationship between schistosomiasis and bladder cancer in the Egyptian population. Epidemiological, experimental, histopathological findings as well as age and gender evidence was drawn by the same authors; leading to a compelling body of evidence that links schistosomiasis of the urinary tract to bladder cancer. Generally, death is mostly due to bladder cancer associated with urinary schistosomiasis and to bleeding from varicose veins in the oesopahagus associated with intestinal schistosomiasis (WHO/TDR, 2003b). In African patients, endemic schistosomiasis appears to be related to a high incidence of not only squamous cell carcinoma, but also undifferentiated tumors and adenocarcinoma of the bladder (Groeneveld *et al.,* 1996).

2.2.5. Schistosomiasis and HIV transmission

STDs increase the probability for HIV transmission, presumably through lesions in the genital mucosa (Plummer *et al.,* 1991; Barongo *et al.,* 1992). Female genital schistosomiasis (FGS), a special form of urinary schistosomiasis due to infection with *S. haematobium*, may be another risk factor for the transmission of HIV in the 44 African countries where these infections coexist. In contrast to many STDs, various lesions in different stages coexist surrounded by an altered epithelium. The lesions vary considerably in number, type and clinical appearance, but they have a common characteristic of bleeding easily, either upon contact or spontaneously. The highest increase in prevalence rates of HIV carrier status during the last 10 years has been observed in Uganda, Kenya, Malawi and The Central African Republic; the prevalence of schistosomiasis heamatobium in these countries accounts for up to 70%. At this stage we cannot valid epidemiological and empirical support for our

hypothesis that female genital schistosomiasis is a co-factor for the spread of HIV in Africa although pathophysiological and immunological grounds such a link seems probable. Reliable prevalence data of female genital schistosomiasis in African countries afflicted by the AIDS epidemic do not exist and correlation between the two infections in different areas cannot be calculated (Feldmeier *et al.,* 1994).

2.2.6. Other pathological manifestations of schistosomiasis

Cutaneous disease is a previously reported but unusual presentation for schistosomiasis. Skin lesions are the sole manifestation and the diagnosis is based on a routine skin biopsy (Davis-Reed and Theis, 2000).

Acute, transient chest radiographic abnormalities and influenza-like symptoms can occur, including cough. In less than 5% of infections, schistosomal egg obstruction of the lung vasculature results in pulmonary hypertension and cor-pulmonale. Limited data suggested that cardiopulmonary schistosomiasis was seen most often in *S. mansoni* infections. Hepatic fibrosis and portal hypertension appear to be a prerequisite to the development of schistosomal cor-pulmonale caused by this species. Although treatment with praziquantel can effectively eradicate all schistosomal infections with minimal toxicity, cardiopulmonary manifestations are not likely to be reversible given the chronic fibrotic tissue changes that are present (Morris and Knauer, 1997).

Brain involvement seen in *S. japonicum* and spinal cord involvement in *S. mansoni* and *S. haematobium* infections were fortunately uncommon (Nash *et al.,* 1982). Schistosomes may reach the central nervous system (CNS) at any time from the moment the worms have matured and the eggs have been laid. For this reason,

CNS involvement may be observed with any of the clinical forms of schistosomal infection. When eggs reach the CNS during the early stages of the infection or during evolution of the disease to its chronic forms, large necrotic-exudative granulomas are found. In-situ egg deposition following the anomalous migration of adult worms appears to be the main, if not the only, mechanism of which *Schistosoma* may reach the CNS in these stages. Most of the cases of CNS involvement associated with the hepatosplenic and cardiopulmonary chronic forms, or with sever urinary schistosomiasis, though more frequent, are asymptomatic (Pittella, 1997).

2.3. Morbidity assessment and diagnosis

Although global estimates of the disease burden exist, the public health impact of schistosomiasis in the field has been poorly evaluated and is still subject to controversies. Apart from few situations where schistosomiasis is or was recognized as an obvious public health problem (as in China, the Philippines, Egypt, Brazil, northern Senegal and Uganda), health personnel and authorities poorly recognize the disease. Because of its unspecific signs and symptoms and its insidious nature, also affected persons often do not perceive it as a serious health problem, which favors the development of late, irreversible sequelae. In many endemic areas, the public health importance of these late sequelae is poorly documented because of the lack of differential diagnostic facilities (WHO/TDR, 2003c).

Clinical manifestations, exposure to risk factors and area endemicity may indicate schistosome infection and define its morbidity presentations. Presence of eggs demonstrated in urine, in case of urinary schistosomiasis, or stool, in case of intestinal schistosomiasis, provides a definitive diagnosis in highly endemic areas. False negative results may appear in areas with low to moderate endemicity. Other direct and/or indirect markers together with parasitological techniques are used for individual or community diagnosis, control programs and for research purposes.

Identifying human schistosome infection is crucial at all stages of schistosome control programmes, and for comparing control programs. Diagnostic methods are the key to such comparisons and, not surprisingly, many tests have been developed over

the years. Complete standardization will never be achieved but, if comparisons are to be justified, the techniques used must be clearly described and their limitations appreciated (Chitsulo *et al.,* 2000).

A specific and direct proof of schistosome infection is the microscopic detection of eggs in urine (*S. haematobium*) or faecal (*S. mansoni*) samples. The eggs are characteristic to each species and easy to identify. They are large (70-180 µm) and have a typical terminal spine (*S. haematobium, S. intercalatum*) or lateral spine (other species). Children with *S. haematobium* nearly always have microscopic or visual blood in their urine (haematuria). Children needing treatment can be also be identified by looking at urine specimens or checking for microscopic blood with chemical reagent strips (WHO/OMS, 1998). Many studies concluded that reagent strip detection of haematuria is useful, valid and rapid diagnostic tool for urinary schistosomiasis. However, sensitivity and specificity vary according to the geographic setting and the age and sex of the study population (Lengeler *et al.,* 1993; Hammad *et al.,* 1997; Lwambo *et al.,* 1997).

2.3.1. Immunodiagnostic techniques

2.3.1.1. Detection of specific antibodies

Serological tests have been described for the demonstration of specific antibodies and their isotypes (Simpson *et al.,* 1990). Despite the high sensitivity and specificity of antibody detection, a serious drawback is the fact that antibody levels generally do not give any information on the intensity of infection, and elevated antibodies are still detectable many years after treatment. Also it is not strong enough to apply this antibody assay for individual diagnosis to differentiate between current and cured infection or for the assessment of cure (Van Lieshout *et al.,* 2000). Immuno-epidemiological studies revealed an association between the levels of specific IgE and resisitance against reinfection after chemotherapy, while susceptibility seemed to be associated with an IgG4 response (Hagan *et al.,* 1991; Dunne *et al.,* 1992). In elderly people (60 and above years old), high levels of

blocking antibodies may be responsible for their increased susceptibility to infection (Satti, 1993; Fulford *et al.,* 1998; Ahmed, 1999).

2.3.1.2. Detection of eosinophils and eosinophil cationic protein (ECP)

Eosinophils are found in large numbers in the schistosome egg granuloma (Eltoum *et al.,* 1989). Compared with haemturia and proteinuria, both found in a variety of conditions, the presence of eosinophils in urine is more specifically related to urinary schistosomiasis although eosinophiluria can be seen in various other conditions as reviewed by Nolan and keller (1988). Results from several studies pointed to ECP, one of the proteins contained in the eosinophil granulocyte, as a promising direct marker of urinary bladder morbidity reflecting the local inflammatory responses of the bladder wall in response to schistosome eggs (Vennervald *et al.,* 2000).

2.3.1.3. Detection of schistosome circulating antigens

Schistosome antigens can be classified, according to the life stage of the parasite into cercarial antigens (Hayunga *et al.,* 1986), adult worm associated antigens (Deelder *et al.,* 1994) and egg antigens (Nour el Din *et al.,* 1994). The detection of these antigens in the circulation of the host potentially offers a large number of diagnostic, epidemiological and research applications (Van Lieshout, 1996). The two antigens that have been extensively studied, circulating anodic antigen (CAA) and circulating cathodic antigen (CCA), belong to the group of the adult worm gut-associated antigens (Qian and Deelder, 1982; Deelder *et al.,* 1994). The development of monoclonal antibody based sandwich ELISA's has made it possible to detect CCA and CAA in a sensitive and high specific manners (Deelder *et al.,* 1989; De Jonge *et al.,* 1990). CCA and CAA were demonstrated in urine and serum of infected individuals with *S. mansoni* and with other *Schistosoma* species, with the exception of *S. mekongi*, which has not been examined so far (Van Lieshout *et al.,* 1992; Kremsner *et al.,* 1993; Kremsner *et al.,* 1994). *S. haematobium* antigens are present in urine from infected individuals and originated both from worms like circulating antigens, CCA and CAA (Deelder *et al.,* 1994) and from *S. haematobium* eggs including SEA

and 29 Kda *S. haematobium* peptide. The detection of SEA in urine indirectly assesses live schistosome eggs in urinary tract or urine (Nibbelling *et al.,* 1998).

2.3.1.3.1. Main characteristics of CAA and CCA

CAA and CCA are glycoconjugates that belong to the group of the adult worm gut-associated circulating antigens. These antigens are released into the circulation of the host by regular regurgitation of the undigested contents of the parasites gut (Van Lieshout, 1996). Both CAA and CCA are genus specific, trichloroacetic acid (TCA) soluble and heat resistant (Nash *et al.,* 1974; Carlier *et al.,* 1975; Deelder *et al.,* 1976). They are also present in the primordial gut cells of cercariae and young worms (Andrade and Sadigurski, 1978). CAA and CCA are extremely stable as illustrated by the detection of CAA in mummy tissue (Deelder *et al.,* 1990). CAA is negatively charged (Berggren and Weller, 1967) and is also known as SCA, GASP and GASCAP (Von Lichtenberg *et al.,* 1974; Nash and Deelder, 1985). CCA is neutral or positively charged (Deelder *et al.,* 1976) and is also known as "antigen M" (Carlier *et al.,* 1975).

2.3.2. Ultrasonography

Ultrasonography (US) is suitable for diagnosing schistosomiasis-related organic pathology and is particularly useful to assess its evolution after therapy and/or interruption of exposure to the *Schistosoma* parasites (Homeida *et al.,* 1988b; Richter, 2000). In urinary schistosomiasis interpretational errors may be due especially to inadequate bladder filling. US changes due to urogenital tuberculosis and bladder malignancies may sometimes resemble those due to schistosomiasis but the clinical presentation is different (Das *et al.,* 1992; Mostafa *et al.,* 1995; Chung *et al.,* 1997). In the case of intestinal schistosomiasis, ultrasonography was found to be a much more sensitive technique than clinical evaluation in estimating the degree of Symmers' fibrosis in a Sudanese population (Homeida *et al.,* 1988a). Inter-observer variance may also occur especially in mild pathology, but is now minimized by the actual standardized methodology (Niamey Working Group, 2000). Further systematic US-studies are

needed not only on the evolution of hepatosplenic and urinary pathology but also on that of intestinal, biliary and genital pathology induced by schistosomiasis, as well as on the influence of schistosomiasis on the outcome of pregnancy (Richter, 2000).

Ultrasound has the advantage of being non-invasive, well accepted by the communities, relatively simple to perform and provide a direct image of the pathological changes due to schistosome infection in the urinary tract (Hatz *et al.,* 1990). It has furthermore proved to be a good method for post treatment follow-up of regression and reappearance of lesions in the urinary tract (Hatz *et al.,* 1998; Wagastoma *et al.,* 1999). However, mild lesions and early inflammatory changes may be missed completely and mild pathology is subjected to variation between observers (Burki *et al.,* 1986; Vennervald *et al.,* 2000).

2.3.3. Questionnaires

Using questionnaires for the diagnosis of schistosomiasis aims at reducing the global burden of the disease, mainly through the large-scale application of chemotherapy. Evidence from interviewed Tanzanian children indicated that reported blood in stools and bloody diarrhoea are valuable indicators for community diagnosis of *S. mansoni*. However, the diagnostic performance of the questionnaires for *S. mansoni* was weaker than for *S. haematobium* (Lengeler *et al.,* 2002).

2.4. Treatment and control

Three safe effective drugs, praziquantel, oxamniquine and metrifonate, are now available for schistosomiasis and are included in the WHO Model List of Essential Drugs. Praziquantel, the drug of choice, is effective against all forms of schistosomiasis with few and only transient side effects. Oxamniquine is used exclusively in some African countries and South America to treat intestinal

schistosomiasis. Metrifonate has proved to be safe and effective for the treatment of urinary schistosomiasis. Even though re-infection may occur after treatment, the risk of developing severely diseased organs is diminished and even reversed in young children. In most areas, a reduction in the overall number of cases is maintained for 18-24 months and in other areas for up to five years without further intervention (WHO/OMS, 1998).

In 1984, the WHO Expert Committee on the Control of Schistosomiasis recommended a strategy for morbidity control, with chemotherapy as the major operational component. Morbidity control strategy can be effective through an integrated approach that includes diagnosis using urine filtration and faecal smear techniques. Serology can be helpful (antigen detection in endemic areas and antibody tests in non-endemic areas). Drug treatment with praziquantel is effective in a single dose against all species and oxamniquine is effective in a single dose but only against *S. mansoni*. Snail control through focal molluscicide; provision of adequate safe water supply and sanitation; and health education are complementary to the control strategy. Significant advances were achieved when health services are strengthened by training microscopists and providing facilities such as vehicles, drugs and microscopes (BNHP, Annual Reports, 1982-1990; WHO, 1993; WHO/TDR, 2003b). Approaches differ for controlling the various forms of schistosomiasis and must be adapted according to the epidemiology, resources and culture of each country. Schistosomiasis control is a long-term commitment. While short-term objectives to reduce prevalence can be achieved (up to 75% within two years in many endemic

areas), surveillance and maintenance must continue for 10-20 years (WHO/OMS, 1998). Figure (2) shows a step-wise approach to schistosomiasis control with chemotherapy as the main operational component, which depends on the epidemiological situation and availability of resources.

Treatment should be available in health facilities for all those who are symptomatic and/or can be diagnosed. Because, in many endemic areas, the health infrastructure may not reach many of those at high risk of schistosomiasis morbidity, treatment coverage can be extended through community or school-based programmes, with or without individual diagnosis. In low prevalence areas, those infected are treated and the strategy should include methods for limiting transmission of schistosomiasis (WHO, 2000a).

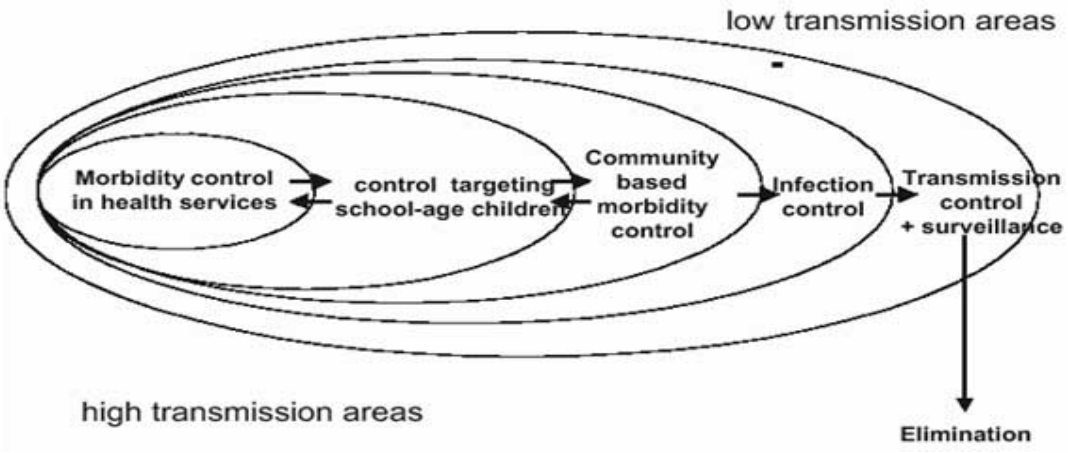


Figure (3): Schema for schistosomiasis control (cited from WHO, 2000a).

Most of research issues needed to address constraints related to schistosomiasis control are related to quantifying the disease burden, rapidly identifying those at risk of morbidity, optimizing available drugs, and reducing

transmission. However, research to bridge the gap between the bench and the field is also needed (WHO/TDR, 2003c).

3. CHAPTER THREE: MATERIALS AND METHODS

3.1. Study design

Females at the childbearing age with certain gynecological complaints were submitted to selection criteria for a case control study. The study investigated cases passing *S. haematobium* eggs in urine and negative controls from Rahad area. Controls from a non-endemic area (Khartoum State) were also included. Due to the far distance of Rahad hospital from the surrounding villages, a mobile clinic was set off to fit the requirements of cases-controls selection.

Samples collection as well as parasitological, clinical, ultrasonography and gynecological examination were conducted in the hospital following the selection of cases and controls. Treatment was secured to all patients as directed by the physician. A sandwich Enzyme-linked immunosorbent assay (ELISA) was used to determine the levels of schistosome circulating antigens in urine, serum and vaginal wash samples. Study outcomes were analyzed using the statistical package for social sciences ver.10 (SPSS10) computer package.

3.2. Study area and study population

Rahad area - North Kordofan State is in the Mid-western Sudan where *S. haematobium* infection is one of the common water-borne diseases. The wide distribution of *Bulinus sp.* snails (the intermediate host for *S. haematobium*) and the high exposure to infection lead to the high prevalence of urinary schistosomiasis. In the area, 30% of the school children suffer from urinary schistosomiasis (Raja′a, 1997), and 18.6% of the females at the childbearing age were found to be positive for *S. haematobium* infection in a preliminary survey previous to this study. Most of the population consisted of Arabs and Nuba while tribes from southern Sudan were minority. See appendix (I) for Sudan map.

3.3. Sample size and selection criteria

EPIINFO6 software was used to calculate the sample size based on the expected frequency of exposure among not ill and ill group and the computer was fed

with the following estimates: 67.6% expected frequency of exposure among not ill group; 91.50% exposure among ill group (difference between the two proportions: 91.50%-67.6%= 25%); 95% confidence interval; 80% power of detection; 5.16 odds ratio; and 2:1 ratio between not ill and ill groups. Based on the previous figures, sample size was calculated and **120** (calculated)-2 females were enrolled in the study consisting of **80** (calculated)+4 controls and **40** (calculated)-6 cases. Controls were recognized as endemic and non-endemic groups based on area of endemicity with schistosomiasis haematobium. Subjects found positive for *S. haematobium* eggs in urine were selected as cases. Others with no reports of urinary schistosomiasis history and found negative for urine examination were selected as controls. Both cases and controls were having gynecological complaints. Virgins were excluded from the study.

3.4. Parasitological methods

Two midday urine samples were collected to coincide with the peak urinary excretion of *S. haematobium* eggs. Syringe filtration technique was performed with reusable nylon mesh (nytrel) filters (WHO document 1983). Collections of 10 milliliters (ml) urine from each sample were withdrawn with a 10ml syringe. The urine was then forced through filter holder containing the mesh filter. The filter inside was removed with a forceps and placed face up on the slide. A drop of normal saline was placed on the edge of the filter to improve visualization of *S. haematobium* eggs present and to prevent dryness of the filter. All the eggs on the filter were counted under the microscope. Intensity of infection was expressed as mean egg count per 10 milliliters urine. Cases positive for *S. haematobium* infection were treated with praziquantel (40mg/kg body weight).

3.5. Questionnaire

A questionnaire was filled for each subject after cases and controls were carefully selected. It included personal information for identification; complaints related to uro-genital tract as well as obstetrical and gynecological history. Parasitological and clinical data were also included. A form of consent was signed by each patient after a detailed explanation of the study and its purpose (Appendix II and III).

3.6. Detection of haematuria, leukocyturia and proteinuria

Haematuria, leukocyturia and proteinuria levels were measured by dipping reagent strips in urine samples for few seconds and then removed. Levels expressed by numbers of crosses (+, ++ or +++) were determined according to the change of color compared to the grades in the reference index of the utilized strips.

3.7. Clinical and gynaecological history and examination

Hemoglobin level (grams/liter) was measured using the "Sahl" apparatus and body weight was measured in kilo-grams. Patients' complaints, past medical, surgical as well as gynecological and obstetrical history were reported and examination was done by a trained physician. Each patient was lying supine and relaxed and a gentle palpation of the entire abdomen and suprapubic was performed to detect splenomegaly, tender abdomen and/or supra-pubic tenderness. The gynecological examination was performed in a relaxing surrounding with the attendance of a female nurse or a chaperone if indicated. Warm instruments and a gentle, unhurried manner with continued explanation and reassurance helped in securing patients' relaxation and cooperation. The knees were slightly flexed and supported as an aid to relaxation of the abdomen muscles and a thorough gynecological examination was carried out with general external inspection, followed by internal inspection to vagina and cervix using a speculum.

3.8. Ultrasonography

Patients were lying supine and relaxed. The examination of schistosomiasis patients with ultrasound requires uncovering of the abdomen and in our study of urinary schistosomiasis also of the pubic area. The pelvis and lower abdomen were scanned and regular intervals of distance were recorded using a scanner of an Aloka SSD-1700, UST-984-5 (5MHz) ultrasound machine (suitable device was used for field purposes). Scanning was performed with the bladder full as described by De Cherney and Pernoll *et al.,* (1994). Dimensions (width and /or length) of vagina and uterus were measured and abnormal echogenicity in both urinary and reproductive tract were reported.

3.9. Collection, preservation and storage of urine, serum and cervico-vaginal secretions samples

Urine samples were collected in wide mouthed containers and few drops of 0.4% [w/v] sodium azide were added to urine as a preservative. Five milliliters blood samples were collected and serum was carefully removed after centrifugation for 10 minutes. Five milliliters of Lithium Chloride-phosphate buffered saline (10 mM LiCl-PBS; PH 7.2) were instilled into the vagina at the beginning of the gynecological examination; the washed secretions were reaspirated into a syringe as described by Belec *et al.* (1995). The samples were placed immediately in thawing ice for less than 3 hours then were centrifuged and supernatant was collected.

All samples (urine, serum and vaginal wash) in 1.5ml vials were kept in liquid nitrogen until transportation from the field. In the central laboratory, samples were stored frozen at –20˚C until use.

3.10. Quantitative determination of circulating cathodic and anodic antigens

A sandwich enzyme linked immunosorbent assay (ELISA) was performed using monoclonal antibodies (McAbs) against schistosome circulating antigens. CCA was measured in urine and vaginal wash samples as described in the modification after De Jong *et al.* (1990). And CAA was measured in serum and vaginal wash samples as described in the modification after Deelder *et al.* (1989). For the preparation of buffers, see (Appendix, IV).

3.10.1. Pretreatment of urine and vaginal wash samples

Before CCA assay was performed, 50 µl of 4% trichloroacetic acid (TCA) were added to 50 µl urine samples in V-bottom ELISA plates. They were mixed well and left for 5 minutes at room temperature. Then, the mixture was centrifuged at 3250 round per minute for 45 minutes until a small pellet was performed. Fifty micro liters supernatant were divided into 25µl duplicates and were added to 25µl neutralization buffer already present in test plate for each duplicate. Final PH was between 6 and 7. Final dilution was ¼. The same procedure was performed for the pretreatment of vaginal wash samples.

3.10.2. CCA assay

Ninety-six wells maxi-sorb microtitration plates were coated by adding 75 µl of 5µg/ml in PBS (1/500 dilution) anti-CCA McAb (Anti-CCA IgG3 MAb 54-5C10-A) each well. The plates were post coated with 4 mg BSA/12 ml PBS, 100 µl/well, to block free sites and hinder nonspecific binding. After the blocking step, the following were added:

- Fifty µl/well samples pretreated with TCA, final dilution ¼ in duplicates.
- Fifty µl/well standard series of adult worm antigen-trichloroacetic acid (AWA-TCA), from 1000 to 0.03 ng/ml (1/√10 dilution, 10x) in CCA buffer.
- Fifty µl/well reference samples including negative and positive controls. Four wells were left empty as blanks.
- Fifty µl/well biotin conjugate of anti-CCA McAb (IgM MAb 8-3C10) diluted in CCA buffer, 1/2000.
- Fifty µl/well alkaline phosphatase conjugate of streptavidin diluted in CCA buffer, 1/3000.
- One hundred µl/well para-nitrophenylphosphate (pNPP) substrat (1 mg/ml diethanolamine (DEA)-buffer).

After each step, a thorough washing was performed with a minimum of 150 ml wash-buffer per plate (4 times); and all steps (except substrate) were performed in a heated shaking incubator system for 15 minutes. After overnight incubation in the dark at 4ºC, the optical densities were measured at 504 nm using ELISA plate reader. The quantitative values of the samples were read against the standard curve after being fitted in a 4-parameter computer curve using Microsoft excel.

3.10.3. Pretreatment of serum and vaginal wash samples

Before CAA assay was performed, an amount of 60µl of 4% trichloroacetic acid (TCA) was added to 60 µl serum samples in V-bottom ELISA plates. They were mixed well and left for 5 minutes at room temperature. The mixture was centrifuged at 35000 round per minute for 4 minutes. Sixty micro liters supernatant were divided equally into duplicates of 30µl that were added to 30µl neutralization buffer already present in test plate for each duplicate. Final PH was between 6.8 and 7.6. Final dilution was ¼. The same procedure was performed for the pretreatment of vaginal wash samples.

3.10.4. CAA assay

The ELISA plates were coated by adding 75 µl of anti-CAA McAb (Anti CAA MAb 120-1B10-A), 1/1100 dilution of 2µg/ml in PBS, to each well. The plates were then post coated with 1/100 dilution of 0.33 mg BSA/ ml in PBS, 100 µl/well. After coating and blocking, the following were added:

- Fifty µl/well samples pretreated with TCA, final dilution ¼ in duplicates.
- Fifty µl/well standard series of AWA-TCA, from 1000 to 0.03 ng/ml (1/√10 dilution, 10x) in CAA buffer.
- Fifty µl/well reference samples including negative and positive controls. Four wells were left empty as blanks.
- Fifty µl/well biotin conjugate of anti-CAA McAb (alkaline phosphatase-conjugated protein A-sepharose-purified mouse Mab 120-1B10-A) diluted in CAA buffer, 1/2000.
- Fifty µl/well alkaline phosphatase conjugate of streptavidin diluted in CAA buffer, 1/3000.
- One hundred µl/well pNPP substrate (1 mg/ml DEA-buffer).

After each step, a thorough washing was performed with a minimum of 150 ml wash-buffer per plate (4 times); and all steps (except substrate) were performed in a heated shaking incubator system for 15 minutes. After overnight incubation in the dark at 4ºC, Optical densities were measured at 504 nm. The quantitative values of the samples were read against the standard curve after being fitted in a 4-parameter computer curve using Microsoft excel.

3.11. Quantitative determination of soluble egg antigen

3.11.1. Pretreatment of samples

Fifty µl/well urine samples and the same quantity of vaginal wash samples were heated for 30 minutes at 70ºC in v-bottom ELISA plates. The samples were directly tested for the SEA quantitative determination (Appendix, IV).

3.11.2. *S. haematobium* SEA assay with 290-2E6 monoclonal

ELISA plates were coated with 50 µl/well anti SEA 290-2E6 McAb at 1/100 dilution in 0.1 M sodium carbonate buffer and incubation went out over night at room temperature. One hundred µl/well of 0.1% bovine serum albumin (BSA) in 0.1 M sodium carbonate buffer (1 mg/ml) were added to block free sites; and plates were incubated at 37ºC for I hour in the shaker. The following were added:

- Fifty µl/well of samples (25µl sample plus 25µl assay buffer making a 1:1 dilution) and references already pretreated and diluted.
- Fifty µl/well standard series of SEA, from 1000 to 0.03 ng/ml. Plates were incubated at 37ºC for 1 hour in the shaker.
- Fifty µl/well biotin conjugate of anti-SEA McAb at 1/1000 dilution in assay buffer with 2% (20 µl/ml) fetal calf serum (FCS). Incubation took place for 1 hour at 37ºC in the shaker.
- Fifty µl/well alkaline phosphatase conjugate of streptavidin diluted in assay buffer, 1/3000. Incubation took place for 30 minutes at 37ºC in the shaker.
- One hundred µl/well pNPP substrat (1 mg/ml DEA-buffer).

After overnight incubation in the dark at 4ºC, the plates were adjusted to room temperature and condense was wiped from the bottom; then optical densities were measured at 504 nm. The quantitative values of the samples were read against the standard curve after being fitted in a 4-parameter computer curve using Microsoft excel. Figure (4) demonstrates the basic form of an ELISA plate used for circulating antigens testing.

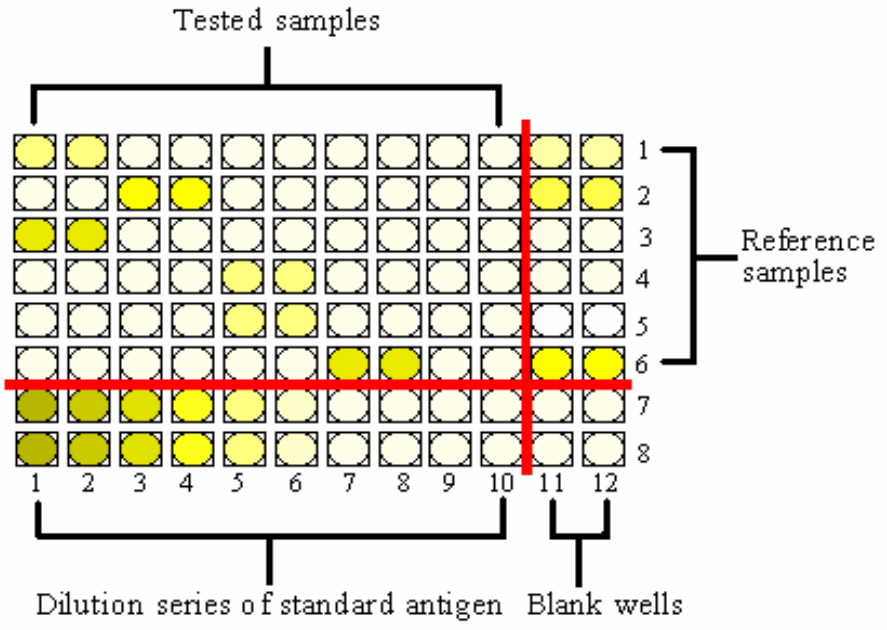


Figure (4): Ninety six wells ELISA plate used for the quantitative determination of schistosome circulating antigens; the dense of yellow colour reflects antigen concentration.

The cut-off value for the CAA assay was 1.3 ng of the TCA-soluble fraction of the adult worm antigen (AWA-TCA) per ml. For the CCA assay the cut-off value was 82 ng- AWA-TCA /ml. and for the SEA assay the cut-off value was 30ng/ml. AWA-TCA contains approximately 3% (w/w) CAA and 3% (w/w) CCA (Van dam *et al.*, 1993).

3.12. Statistical analysis

Statistical package for social sciences version10 (SPSS10) was used for data analysis. Parametric tests were used when measurements were normally or log normally distributed. Non-parametric tests were used to analyze non-normalized data. Chi square test was used to analyze qualitative variables.

4. CHAPTER FOUR: RESULTS

4.1. Study groups

In total, 118 females at the childbearing age (15-50 years old) were enrolled in a case control designed study. Thirty-four (28.8%) were selected as cases passing *S. haematobium* eggs in urine from Rahad area and 84 (71.2%) were selected as controls, of whom 45 (38.1%) were from Rahad area and 39 (33.1%) were from Khartoum area (1:2 cases to controls ratio). The mean ages for study groups were 24.7 years for cases from Rahad (standard error SE= 1.29, standard deviation SD= 7.5, range: 15-45); 28.6 years for controls from Rahad (SE=1.1, SD=8, range: 17-50); and 34.5 years for controls from Khartoum (SE=1.1, SD= 6.6, range: 20-50) respectively. Pregnant women represented a percentage of 23.5% among cases and 8.9% among controls from Rahad.

4.2. Parasitological examination

4.2.1. Statistical analysis

S. haematobium egg count was log normally distributed and results were expressed as geometric means. Parametric analyses were used to test differences in geometric means of egg count between age groups. Correlations with other measurements were tested and are described later in this chapter.

4.2.2. Detection of *S. haematobium* eggs in urine

Qualitative and quantitative detection of *S. haematobium* eggs (sheg) was performed under the microscope as described previously. Of all the 34 positive cases, 41.2% had past history of schistosomiasis haematobium infection with a duration that ranged from recently being infected, mostly in young cases, to 28 years as maximum duration of infection. Few cases were treated several times during the course of infection. The intensity of infection, expressed as geometric mean, was generally low (6.3 eggs/10 ml urine; SD: 2.19) and ranged from 2 to 61.7 eggs/10ml urine. As

shown in figure (5), the highest egg count was found among the younger age groups and the lowest egg count was demonstrated among the group of 27-32 years old.

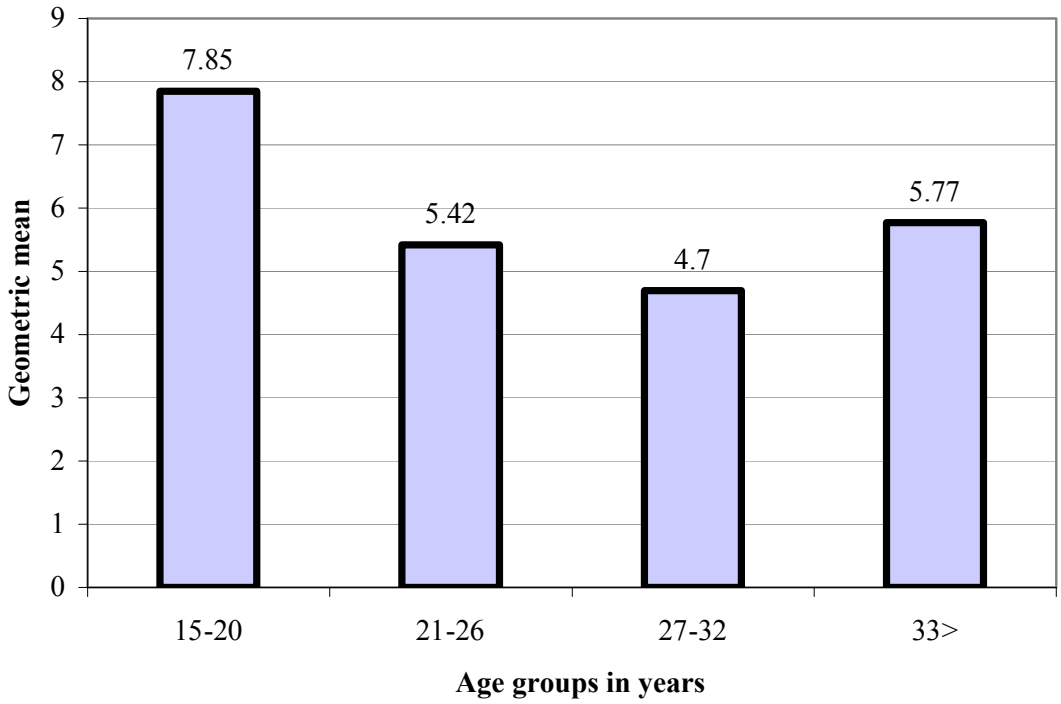


Figure (5): Geometric means of *S. haematobium* egg counts in urine (sheg) between age groups among infected females from Rahad area.

4.3. Reagent strips, hemoglobin level and body weight

4.3.1. Statistical analysis

Chi-square (χ^2) was used to test differences, expressed as χ^2 P-values, in the prevalence of macrohaematuria, microhaematuria, leukocyturia and proteinuria among females infected with *S. haematbium* and negative controls from Rahad area. Differences were also tested between age groups. One-way ANOVA was used to test differences in body weights and hemoglobin levels between cases and controls. Pearson correlation between log transformed egg counts in urine and body weight as well as hemoglobin level was also tested.

4.3.2. Measurement of haematuria, leukocyturia and proteinuria in urine

Blood in urine (macrohaematuria), microhaematuria, leukocyturia and proteinuria were tested as indirect markers for schistosomiasis haematobium infection. Although outcomes of all markers varied considerably between cases and endemic

controls, only macrohaematuria and microhaematuria differed significantly between the infected and non-infected groups (Table, 1).

Table (1): Prevalence of macrohaematuria, microhaematuria, leukocyturia and proteinuria among cases and endemic controls from Rahad area.

	Macrohaematuria%	Microhaematuria%	Leukocyturia%	Proteinuria%
Cases	44.1 (15/34)	55.9 (19/34)	44.1 (15/34)	70.6 (24/34)
Controls	0.0 (0/45)	21.4 (9/42)	33.3 (14/42)	69.0 (29/42)
Sig.	-	0.002**	0.3	0.8
OR	-	4.6	1.6	1.1
95%CI	-	1.7-12.6	0.6-4.0	0.4-2.9

Sig.: significance of difference OR: odds ratio
** highly significant, P<0.01. CI: confidence interval.

When testing differences between age groups, prevalence of microhaematuria in females aged from 27 to 32 years was found to be significantly higher among cases than controls from Rahad area (Table, 2). Macrohaematuria, leukocyturia and proteinuria showed no significant association with different age groups, neither in cases nor in controls.

Table (2): Prevalence of microhaematuria between different age groups among cases and controls from Rahad area.

		15-20 years	21-26 years	27-32 years	33> years
Microhaematuria%	**Cases**	53.3 (8/15)	60.0 (6/10)	75.0 (3/4)	40.0 (2/5)
	Controls	36.4 (4/11)	28.6 (2/7)	15.4 (2/13)	9.1 (1/11)
	Sig.	0.4	0.2	0.02*	0.1

* difference is significant, P< 0.05.

4.3.3. Body weight and hemoglobin level

Mean body weight was found to be 54.65 kg (95% CI: 50.66-58.64 kg) for cases, 56.39 kg (95% CI: 52.81-59.97 kg) for endemic controls, and 66.09 kg (95% CI: 62.1-70.08 kg) for non-endemic controls. Body weights of the non-endemic controls were significantly higher than body weights of both cases and endemic controls ($P<0.01$ in both comparisons). Mean hemoglobin level was 11.02 g/liter (95% CI: 10.47-11.58) for cases, 9.68 g/liter (95% CI: 9.27-10.08 g/liter) for endemic controls, and 11.57 g/liter (95% CI: 11.16-11.98 g/liter) for non-endemic controls. The endemic controls had the lowest hemoglobin levels that varied significantly from both cases and non-endemic controls ($P<0.01$ in the two comparisons). For those who were passing *S. haematobium* eggs in urine, no significant correlation was found between intensity of infection neither with body weight nor with hemoglobin level.

4.4. Gynecological history and examination

4.4.1. Statistical analysis

All qualitative data was expressed as prevalence and Chi-square (χ^2) was used to test differences in gynecological and obstetrical data collected from cases, endemic controls and non-endemic controls.

4.4.2. Gynecological and obstetrical history

Gynecological and obstetrical history was carefully reported. Cases and controls from endemic and non-endemic areas were relatively similar in ages when they started menstruating (age at menarche). No significant differences were present when comparing interval between periods and duration of flow (katamina) or number of times each conceived or got pregnant (gravidity). Contraception use was significantly higher among controls selected from Khartoum (non endemic) area. Percentages of subjects having menstrual disorders and period associated with clots were higher among non-endemic controls but the difference was not significant with the other groups. Percentages of subjects with amenorrhea and irregular cycle were higher among cases and controls from Rahad with no significant differences. Only one subject from non-endemic area reported an intermenstrual bleeding. Reports of post coital complaints were very few. Lower abdominal pain, back pain, pruritis, irregular menstrual cycle and dysmenorrhoea were higher in controls from Rahad (Table, 3). Only 3 subjects reported past history of ectopic pregnancy and 2

reports of sexually transmitted diseases were among controls from Rahad. Past history of surgical intervention was higher among controls from Khartoum and varied significantly from cases and controls from Rahad (P<0.01 in both comparisons).

Table (3): prevalence of some gynecological findings among cases, endemic controls and non-endemic controls.

	Cases	**Endemic controls**	**Non-endemic controls**	**Sig.**
Lower abdominal pain%	70.6 (24/34)	84.4 (38/45)	71.8 (28/39)	0.2
Back pain%	58.8 (20/34)	84.4 (38/45)	59.0 (23/39)	0.01*
Pruritis%	14.7 (5/34)	20.0 (9/45)	15.4 (6/36)	0.7
Irrugular menstrual cycle%	38.2 (13/34)	44.4 (20/45)	35.9 (14/39)	0.7
Dysmenorrhoea%	38.2 (13/34)	77.8 (35/45)	59.0 (23/39)	0.002**

Sig.: significance of difference (* P<0.05; ** P<0.01)

4.4.3. Gynecological examination

Physical examination of the pubic and gynecological examination with an external inspection was carried out followed by internal inspection to vagina and cervix using a speculum. Abnormal findings were very few, and comparisons between groups showed no significant differences (Figure, 6).

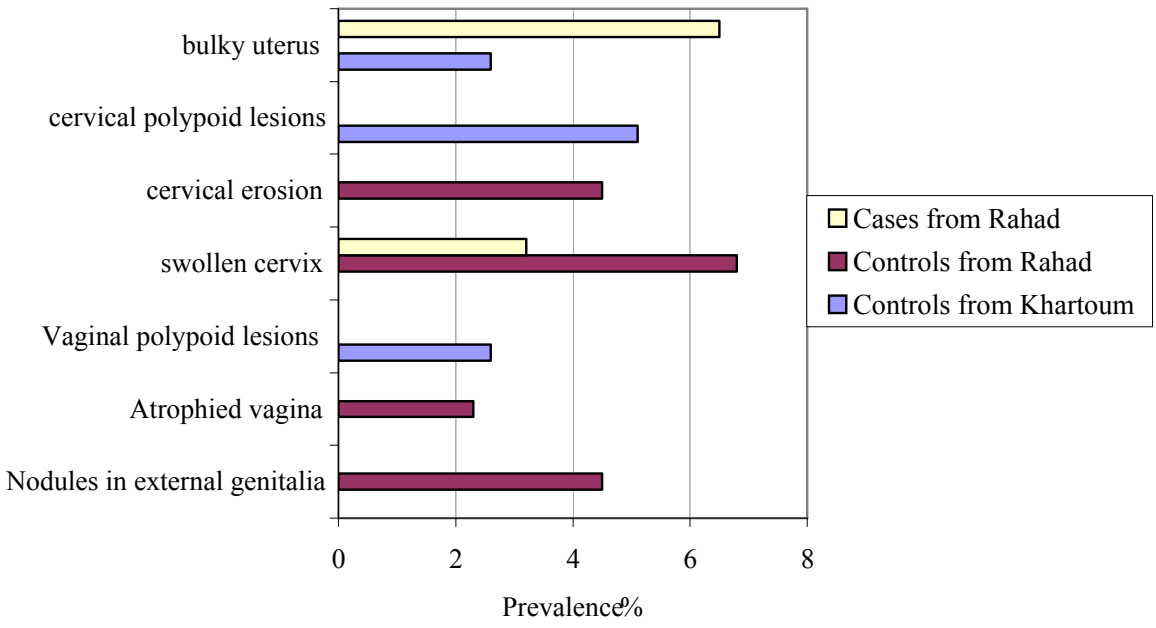


Figure (6): Prevalence of abnormal gynecological findings among cases, endemic controls and non-endemic controls.

4.5. Determination of abnormal echogenicity

4.5.1. Statistical analysis

Chi-square (χ^2) was used to test differences in pathological patterns scanned in pelvis among females infected with *S. haematbium* and controls from endemic (Rahad) and non-endemic (Khartoum) areas. Cervix width as well as uterus width and length were normally distributed and appropriate test was used to examine correlations.

4.5.2. Ultrasonography

Cases positive for *S. haematobium* infection in urine and controls from endemic area (Rahad) and non-endemic area (Khartoum) were subjected to ultrasonographic examination of the general pelvis and more indepth in bladder, cervix, uterus, fallopian tubes and ovaries. The pathology scanned in pelvis was categorized into that found in general pelvis, urinary tract, genital tract and pathology found in both urogenital tracts of the same individual. Higher prevalence of pathology was found among cases and analysis revealed significant differences in prevalence between cases and controls as shown in table (4).

Table (4): Prevalence of abnormal echogenicity scanned in general pelvis including urinary tract, genital tract and urogenital tracts among cases, endemic controls and non-endemic controls.

	General pelvis	**Urinary tract%**	**Genital tract%**	**Urogenital tract%**
Cases	79.4 (27/37)	38.2 (13/34)	61.8 (21/34)	20.6 (7/34)
Endemic controls	55.6 (25/45)	6.7 (3/45)	53.3 (24/45)	4.4 (2/45)
Non-endemic controls	33.3 (13/39)	0.0 (0/39)	33.3 (13/39)	0.0 (0/39)
Sig.	0.000**	0.000**	0.04*	0.003**

Sig.: significance of difference
* difference is significant (P<0.05)
** difference is highly significant (P<0.01)

Significant differences in the pathology of the general pelvis existed in all comparisons. In urinary tract, differences in pathology were significant between cases and endemic controls while all the non-endemic controls showed normal scans. A significant difference was found between cases and non-endemic controls in pathology found in genital tract. Prevalence of individuals presenting pathology in both urinary and genital tracts was significantly higher in cases when compared to endemic controls (Table, 5).

Table (5): Differences expressed as P-values of abnormal echogenicity in pelvis between cases, endemic controls and non-endemic controls.

		General pelvis	Urinary tract%	Genital tract%	Urogenital tract%
Cases/endemic controls	**Sig.**	0.02*	0.001**	0.4	0.02*
	OR	3.0	8.7	-	5.6
	95% CI	1.1-8.5	2.2-33.8	-	1.1-28.8
Cases/nonemdemic controls	**Sig.**	0.00**	-	0.01*	-
	OR	7.7	-	3.2	-
	95% CI	2.7-22.4	-	1.2-8.4	-
Endemic	**Sig.**	0.04*	-	0.06	-

controls/nonendemic controls	**OR**	2.5	-	-	-
	95% CI	1.0-6.0	-	-	-

Sig.: significance of difference (* P<0.05; ** P<0.01).
OR: odds ratio
CI: confidence interval

The pathology scanned by ultrasound in bladder, cervix, and uterus was prevalent among cases and differed significantly when compared to controls from endemic and non-endemic areas (P= 0.001**, 0.01** and 0.03* respecively). An exception was the pathology found in the uterus that showed significant difference with the non-endemic controls while the endemic controls were not significantly different from cases. Pathological findings in ovaries were more prevalent among cases followed by endemic controls and non-endemic controls (P= 0.7). The endemic controls had the highest prevalence of pathology scanned in fallopian tubes followed by cases and endemic controls (P= 0.3). Differences between groups are illustrated in figure (7) and are detailed in table (6).

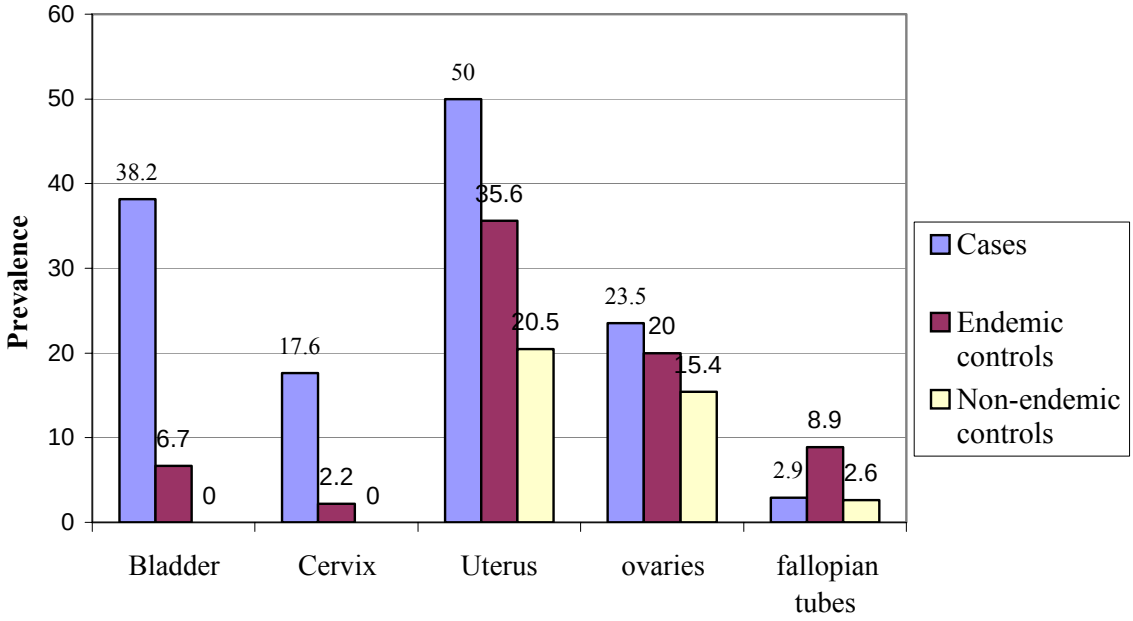


Figure (7): Prevalence of abnormal echogenicity in specific organs among cases and controls from endemic and non-endemic areas.

Table (6): Differences expressed as P-values of abnormal echogenicity in specific organs between cases, endemic controls and non-endemic controls.

		Bladder	Cervix	Uterus	Ovaries	Fallopian tubes
Cases/endemic controls	**Sig.**	0.001**	0.01*	0.2	0.7	0.3
	OR	8.7	9.4	-	-	-
	95% CI	2.2-33.8	1.1-82.5	-	-	-
Cases/non-endemic controls	**Sig.**	-	-	0.008**	0.4	0.9
	OR	-	-	3.9	-	-
	95% CI	-	-	1.4-10.8	-	-
Endemic controls/non-endemic controls	**Sig.**	-	-	0.1	0.5	0.2
	OR	-	-	-	-	-
	95% CI	-	-	-	-	-

Sig.: significance of difference (* P<0.05; ** P<0.01)
OR: odds ratio

CI: confidence interval

Pathological patterns characteristic to the bladder, cervix and uterus and differences between study groups are described in details. Only one subject reported salpingitis among cases and another one among non-endemic controls. One report of adnexeal swelling was found among the endemic controls. Single cases of splenomegaly and hepatomegaly were reported among cases. Table (7) illustrates the prevalence of pathology found in the bladder that includes thickening of the bladder wall, sandy patches, polyps and calcification. No reports of any of the previously mentioned pathological features were found among the non-endemic controls and significant difference in bladder sandy patches was found between cases and endemic controls.

Table (7): prevalence of abnormal echogenicity scanned in the bladder of cases, endemic controls as well as non-endemic controls.

	Thickened bladder wall%	**Sandy patches%**	**Polyps%**	**Calcification%**
Cases	0.0 (0/34)	23.5 (8/34)	2.9 (1/34)	11.8 (4/34)
Endemic controls	2.2 (1/45)	2.2 (1/45)	0.0 (0/45)	2.2 (1/45)
Sig.	-	0.003**	-	0.08
OR	-	13.5	-	-
95% CI	-	1.6-114.5	-	-

Sig.: significance of difference (*P<0.05; ** P<0.01)

Generally, abnormal echogenic findings in the cervix were very few. Four of the cases were presented with bulky cervix, another case had cervical fibroid and one case with calcified nodules in the cervix. Only 1 control from the endemic area reported bulky cervix and non of the 39 controls from non-endemic area reported any of these presentations. Although differences were not significant between cases and controls in the pathology scanned in uterus, some of the findings (retroverted uterus, hypoplastic uterus and thick endometrium) were more frequent among cases and some (bulky uterus, endometrial hyperplasia and uterian fibroid) were more frequent among the endemic controls (Table, 8).

Table (8): Prevalence of abnormal echogenicity scanned in uterus of cases positive for *S. haematobium* eggs in urine and controls from endemic (Rahad) and non endemic (Khartoum) areas.

	Retroverted%	Bulky%	Thick endometrium%	Endometrial hyperplasia%	Mass%	Fibroid%
Cases	8.8 (3/34)	0.0 (0/34)	20.6 (7/34)	0.0 (0/34)	2.9 (1/34)	11.8 (4/34)
Endemic controls	4.4 (2/45)	2.2 (1/45)	11.1 (5/45)	4.4 (2/45)	0.0 (0/45)	15.6 (7/45)
Non-endemic controls	2.6 (1/39)	0.0 (0/39)	10.3 (4/39)	0.0 (0/39)	2.6 (1/39)	5.1 (2/39)
Sig.	0.4	-	0.3	-	0.5	0.3

Sig.: significance of difference (P> 0.05)

4.6. Measurement of schistosome circulating antigen levels

4.6.1. Statistical analysis

Since the data was not normally or log normally distributed for the concentration of CCA, CAA and SEA levels, non-parametric tests were used for analysis and results were expressed as ranges and mean ranks. Kruskal-Wallis test was applied for comparisons between the study groups. Mann-Whitney test was used for the comparisons between each two groups. Chi-square test was used to determine sensitivities and specificities for each assay. Spearman test was used to test non-parametric correlations.

4.6.2. CCA, CAA and SEA levels

CCA, CAA and SEA levels were measured in urine, serum and vaginal wash samples as ng AWA-TCA per milliliter sample for CCA and CAA and ng/ml sample for SEA. Thirty percent of AWA-TCA consists of CCA and of the same value for CAA. Significant differences between cases and controls were found in vaginal wash CCA level, serum CAA level, and urine SEA level (Table, 9).

Table (9): CCA, CAA and SEA levels among cases and controls from endemic and non endemic areas.

		u-CCA	v-CCA	s-CAA	v-CAA	u-SEA	v-SEA
Cases	***Range***	76	1299	67	7	1641	79
	MR	56.4	61.1	68.0	52.8	72.1	59.8
	N	33	30	32	30	33	30
Endemic controls	***Range***	752	281	294	5	10	22
	MR	59.2	64.8	55.5	58.1	52.2	54.8
	N	43	43	45	43	45	43
Non-endemic controls	***Range***	820	589	59	7	5	33
	MR	61.1	43.8	54.2	57.7	55.8	55.8
	N	39	39	39	39	39	39
	Sig.	0.8	0.009**	0.01*	0.6	0.005**	0.8

u: urine; s: serum and v: vaginal wash.
MR: mean rank; N: number of subjects; * P<0.05; ** P<0.01

The CCA level in vaginal wash was found to be significantly higher among cases than non-endemic controls and higher among endemic controls than non-endemic controls. The levels of serum CAA as well as urine SEA were significantly higher in cases than in both endemic and non-endemic controls (Table, 10).

Table (10): Differences expressed as P-values of different schistosome antigen levels between each two groups (cases positive for *S. haematobium* eggs in urine, endemic controls and non-endemic controls).

	u-CCA	**v-CCA**	**s-CAA**	**v-CAA**	**u-SEA**	**v-SEA**
cases/ endemic controls	0.8	0.6	0.02*	0.3	0.003**	0.5
cases/ non-endemic controls	0.5	0.03*	0.009**	0.4	0.02*	0.6
endemic controls/ non-endemic controls	0.8	0.003**	0.8	0.9	0.4	0.9

u: urine; s: serum and v: vaginal wash.
* difference is significant (P<0.05)
** difference is highly significant (P<0.01)

4.6.3. Sensitivities and specificities of schistosme antigens detection assays

Assays' sensitivities and specificities were determined based on the presence of *S. haematobium* eggs urine. the lowest detection level of CAA in serum (5 ng AT/ml) corresponded to 4 eggs/10ml urine; 1 ng AT/ml vaginal wash CAA corresponded to 1 egg/10ml urine; 1 ng AT/ml urine CCA corresponded to 4 eggs/10ml urine; 3 ng AT/ml vaginal wash CCA corresponded to 4 eggs/10ml urine; 1 ng /ml urine SEA corresponded to 2 eggs/ 10ml urine and similarly, 1 ng /ml vaginal wash SEA corresponded to 2 eggs/10ml urine. The cut-off values for CAA, CCA and SEA were 1.3 ng-AT/ml, 82ng-AT/ml and 30ng/ml respectively. The highest sensitive assay was the CAA determination in serum samples, followed by SEA determination in urine samples, CCA determination in vaginal wash samples,

SEA determination in vaginal wash samples and CAA determination in vaginal wash samples. The least sensitive assay was the CCA determination in urine samples that resulted no positive reaction. Specificities were higher in the samples tested from endemic controls and were lower in the non-endemic controls samples (Table, 11).

Table (11): Sensitivities of CCA, CAA and SEA as determined in cases; and specificities as determined in controls from endemic and non-endemic areas.

	Sensitivity%	**Specificity%[a]**	**Specificity%[b]**
u-CCA	0.0 (0/33)	91.1 (41/45)	94.9 (37/39)
v-CCA	16.7 (5/30)	83.7 (36/43)	94.9 (37/39)
s-CAA	31.3 (10/32)	91.1 (41/45)	92.3 (36/39)
v-CAA	6.7 (2/30)	83.7 (36/43)	82.1 (32/39)
u-SEA	21.2 (7/33)	100 (45/45)	100 (39/39)
v-SEA	10.0 (3/30)	100 (43/43)	97.4 (38/39)

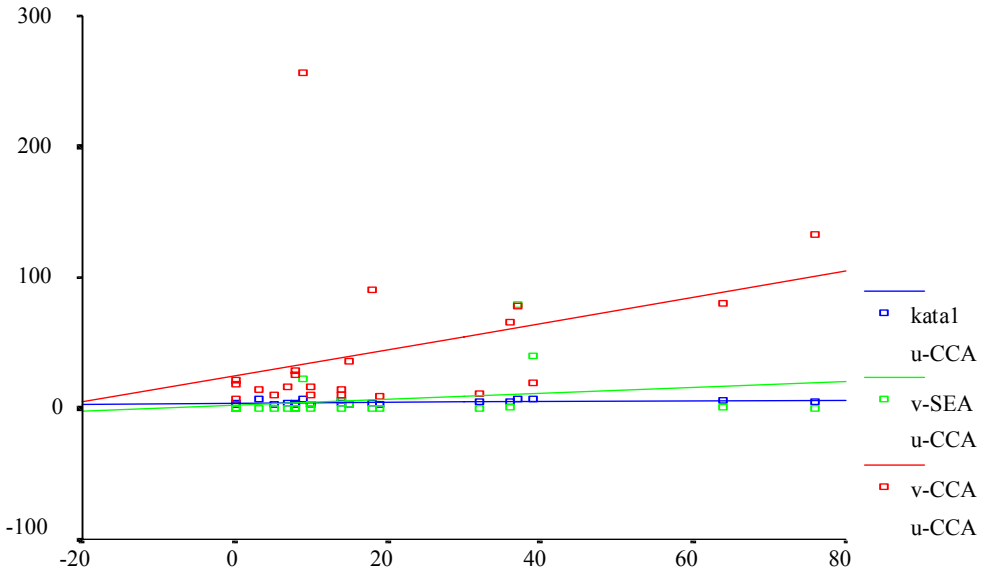
u: urine; v: vaginal wash; and s: serum.
[a] specificity as determined in Rahad area (endemic for *S. haematobium)*.
[b] specificity as determined in Khartoum area (non endemic for *S. haematobium)*.

4.6.4. Correlation with parasitological, clinical and gynecological findings

4.6.4.1. Antigen correlations among cases passing eggs in urine

CCA level measured in urine and vaginal wash, CAA level measured in serum and vaginal wash as well as SEA level measured in urine and vaginal wash showed significant correlations with parasitological, clinical and gynecological measurements among cases passing eggs in urine. Urine SEA levels were significantly higher among cases with positive haematuria in urine (P= 0.04). Urine CCA showed positive correlation with vaginal wash CCA, with vaginal wash SEA and with duration of menstrual flow. Vaginal wash CCA showed negative correlation with age and uterus length, and also showed a positive correlation with serum CAA (Figure, 8: A and B).

(A)



(B)

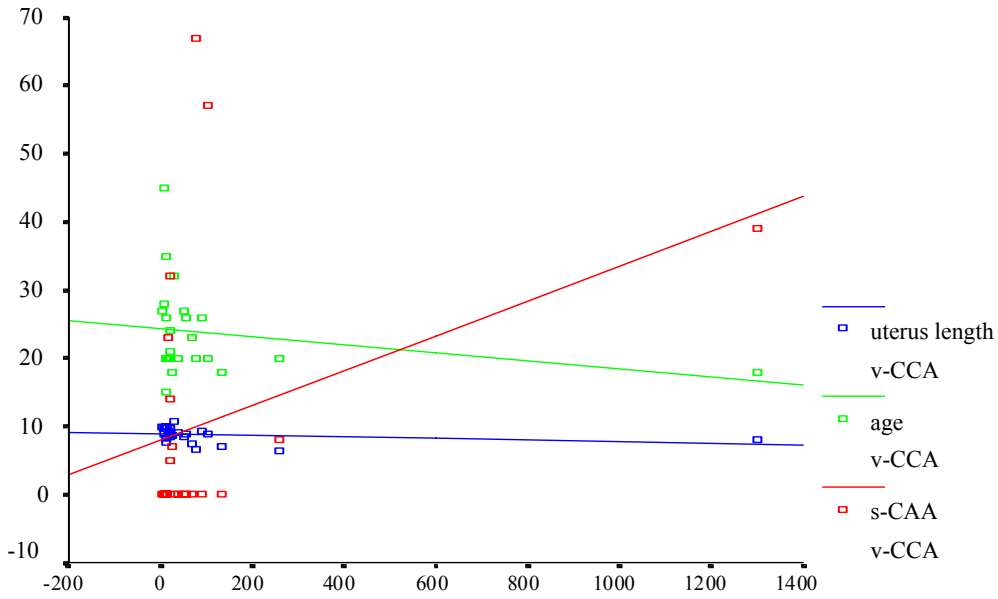
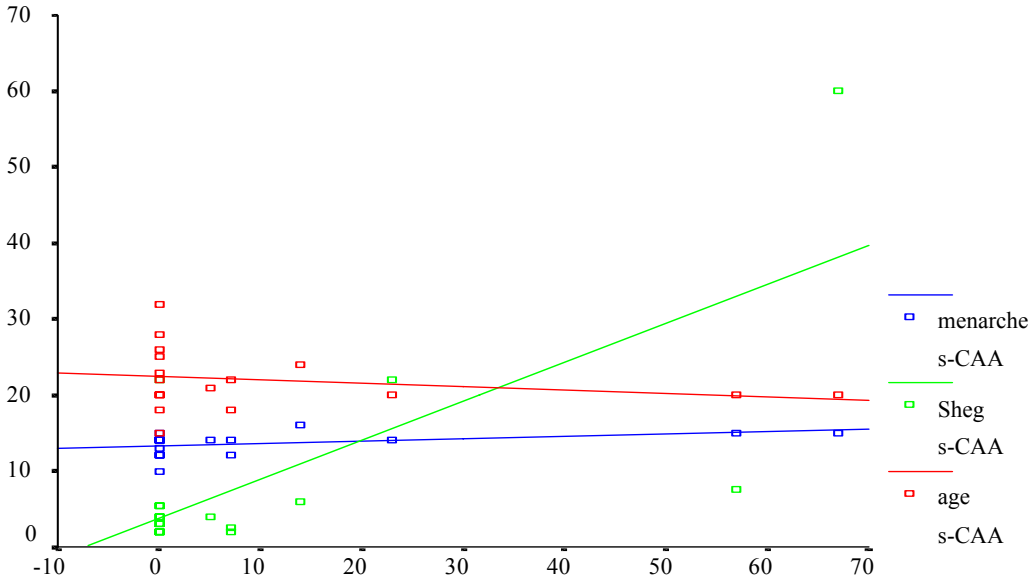


Figure (8): Correlation coefficients among cases for **(A)** urine CCA with: vaginal wash CCA, P= 0.04; vaginal wash SEA, P= 0.03 and kata1 or duration of menstrual

flow in days, P= 0.01 **(B)** vaginal wash CCA with: serum CAA, P= 0.02; age in years, P= 0.03; uterus length (cm) detected by ultrasound, P= 0.005.

Cases also showed positive correlation of serum CAA with age at menarche and *S. haematobium* egg count in urine (sheg) and a negative correlation of serum CAA with age in years; vaginal wash CAA showed a negative correlation with length of uterus (Figure, 9: C and D).

(C)



(D)

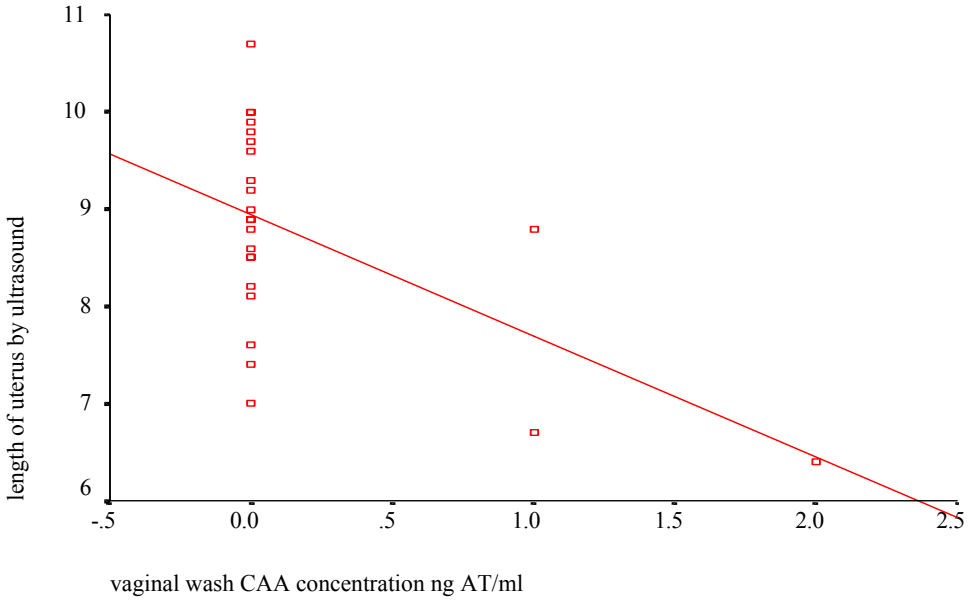
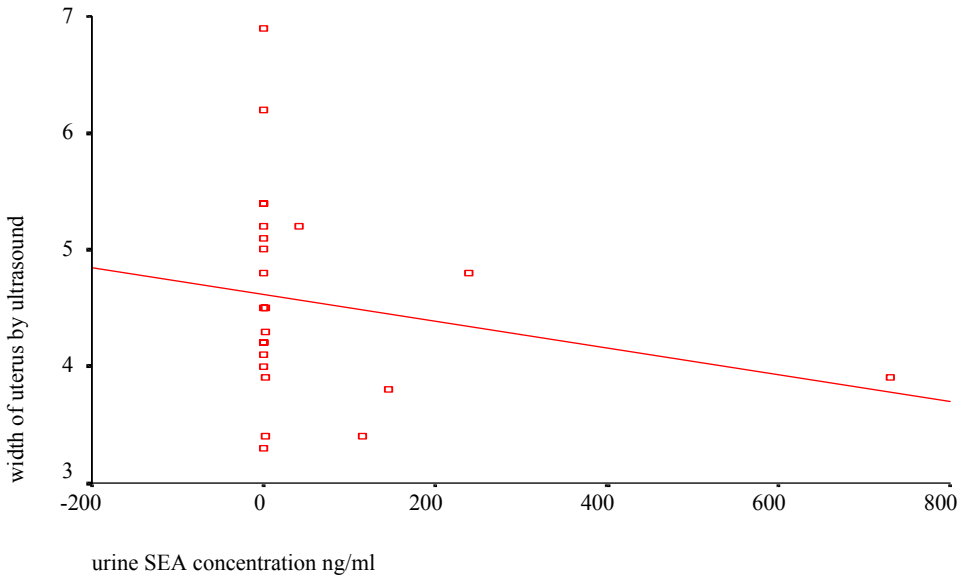


Figure (9): Correlation coefficients among cases for **(C)** serum CAA with: age in years, P= 0.02; *S. haematobium* egg count or sheg, P= 0.008 and age at menarche in years, P= 0.04 **(D)** vaginal wash CAA with uterus length (cm) detected by ultrasound. P= 0.03.

Cases showed negative correlation for urine SEA with uterus width and positive correlation for vaginal wash SEA with age at menarche. Moreover, negative correlations for vaginal wash SEA with uterus length and width was found (Figure, 10: E and F).

(E)



(F)

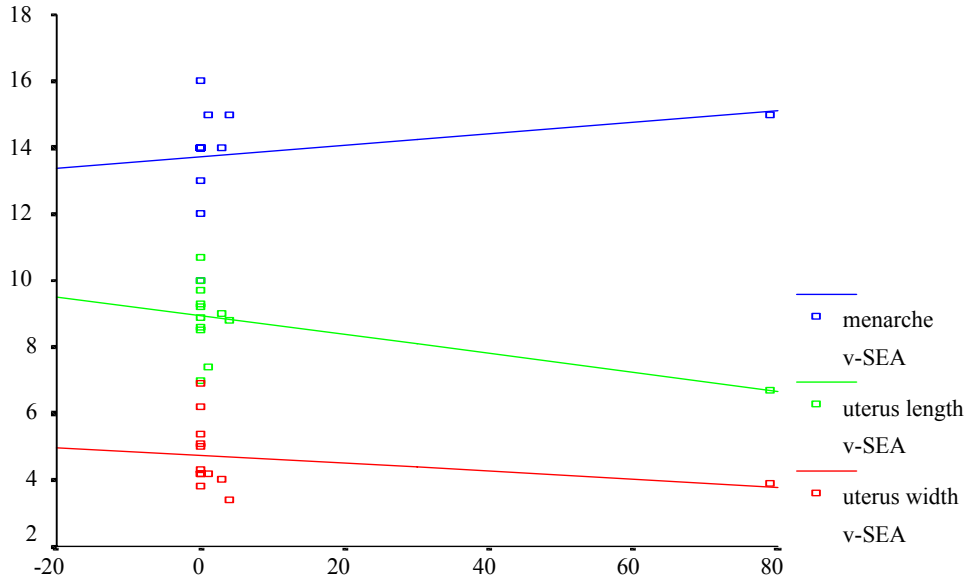
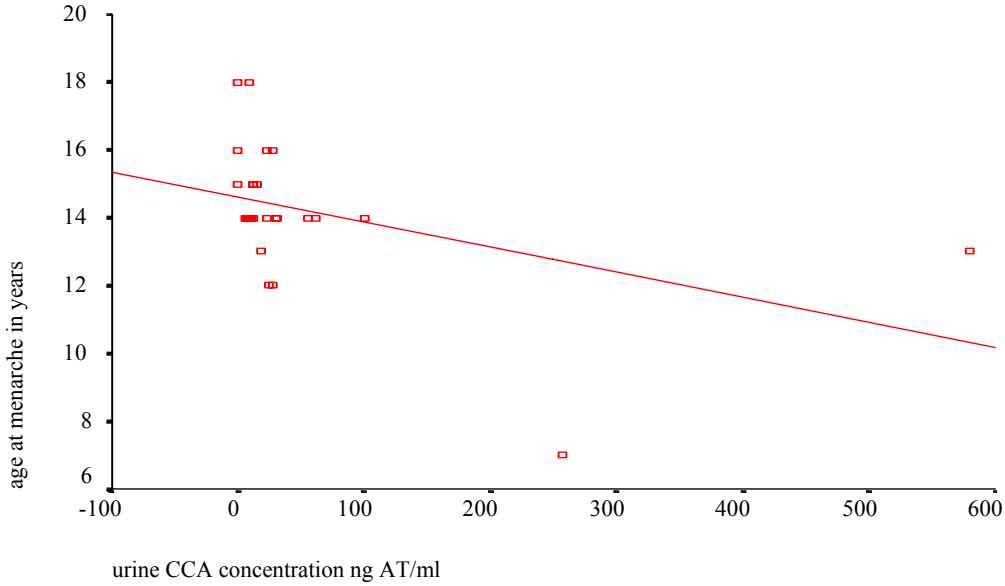


Figure (10): Correlation coefficients among cases for **(E)** urine SEA with uterus width (cm) detected by ultrasound, P= 0.04 **(F)** vaginal wash SEA with: age at menarche in years, P= 0.02; uterus width (cm) detected by ultrasound, P= 0.03; uterus length (cm) detected by ultrasound, P= 0.02.

4.6.4.2. Antigen correlations among controls from endemic area

Endemic controls showed negative correlation for urine CCA with age at menarche. Vaginal wash CCA showed Positive correlation with vaginal wash SEA and negative correlation with cervix width (Figure, 11: A and B).

(A)



(B)

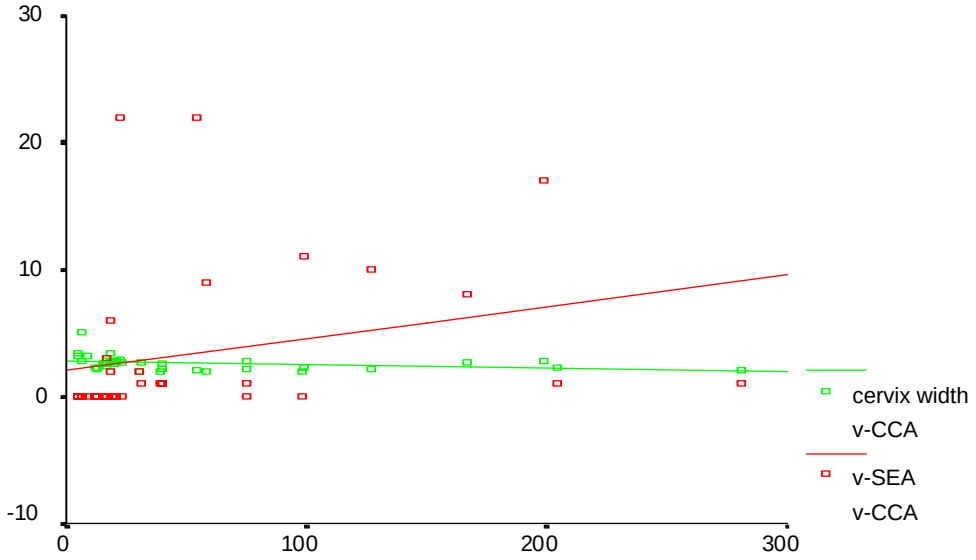
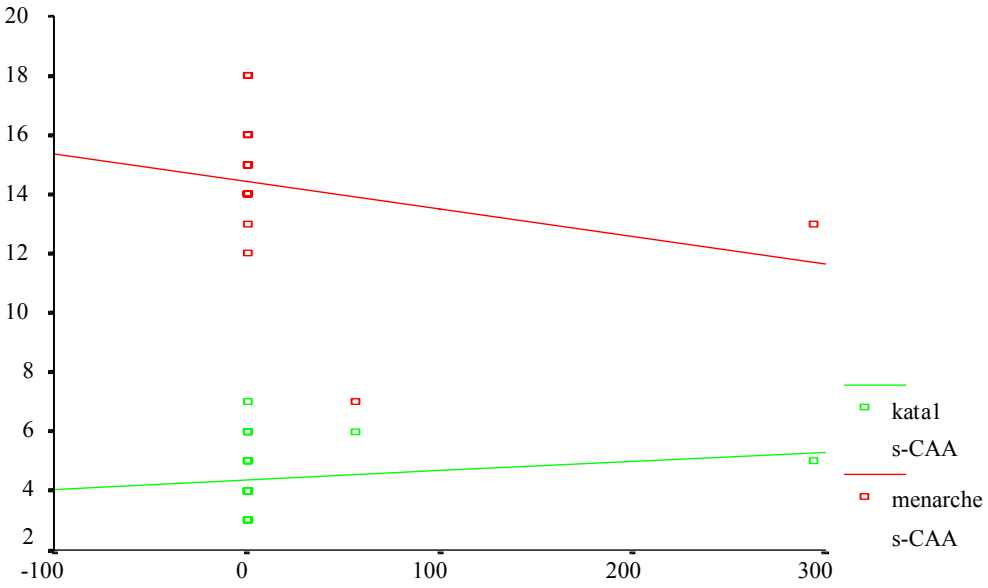


Figure (11): Correlation coefficients among endemic controls for **(A)** urine CCA with age at menarche in years, P= 0.005 **(B)** vaginal wash CCA with: vaginal wash SEA, P= 0.000; cervix width (cm) detected by ultrasound, P= 0.001.

Serum CAA in endemic controls showed a negative correlation with age at menarche and a positive correlation with duration of menstrual flow; and vaginal wash CAA showed a positive correlation with uterus length (Figure, 12 C and D).

(C)



(D)

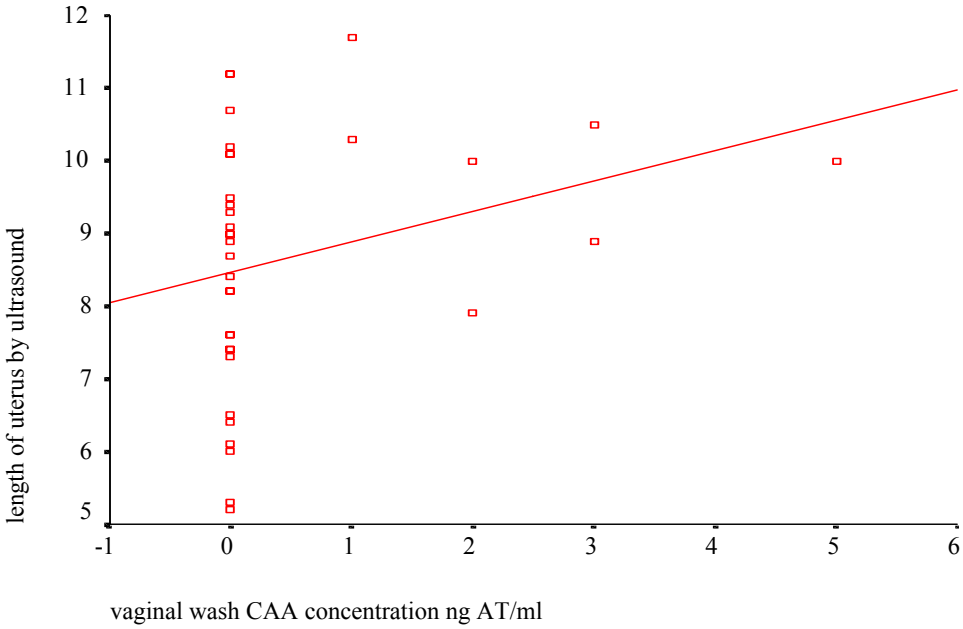


Figure (12): Correlation coefficients among endemic controls for **(C)** serum CAA with: age at menarche in years, P= 0.02; and , kata1 or duration of menstrual flow in days, P= 0.04 **(D)** vaginal wash CAA with uterus length (cm) detected by ultrasound, P= 0.04.

4.6.4.3. Antigen correlation among controls from non-endemic area

The group of the non-endemic controls showed no significant correlations with any of the clinical data. Urine CCA showed a positive correlation with serum

CAA (P= 0.004); and vaginal wash CCA showed a positive correlation with vaginal wash SEA (P= 0.001).

4.6.5. Age-dependent schistosome antigen levels

4.6.5.1. Age-dependent antigen levels among cases passing eggs in urine

Antigen levels, expressed as mean ranks, showed an age related pattern characteristic to CCA level measured in vaginal wash samples among cases passing *S. haematobium* eggs in urine. Vaginal wash CCA showed higher mean rank compared to the other antigens. The curve declined gradually in older age groups. The other antigens investigated were relatively stable in all age groups (Figure, 13).

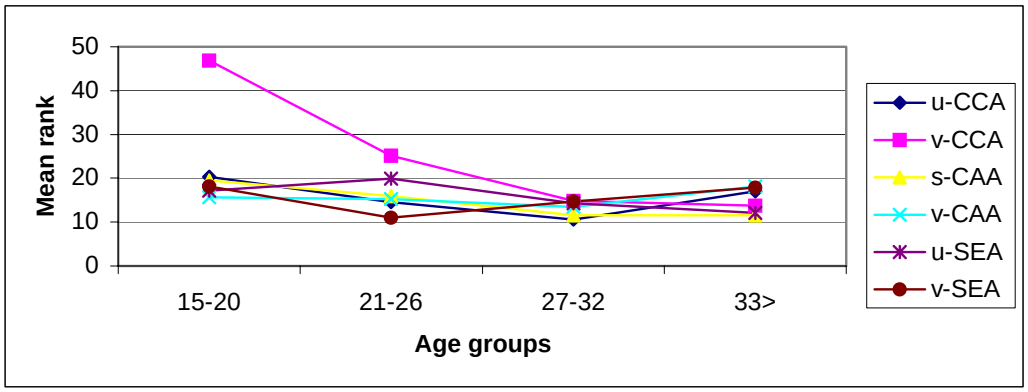


Figure (13): Age related mean rank of schistosome antigen levels measured in urine, serum and vaginal wash samples from cases passing *S. haematobium* eggs in urine.

4.6.5.2. Age-dependent antigen levels among controls from endemic area

Similar to cases, endemic controls also showed relatively stable antigen levels among age groups with the exception of CCA level in vaginal wash samples that presented a higher mean rank in the 15-20 years age group compared to other antigens and increased rapidly in the 21-26 years age group. Vaginal wash CCA level decreased in the 27-32 years age group and again increased in older females (Figure, 14).

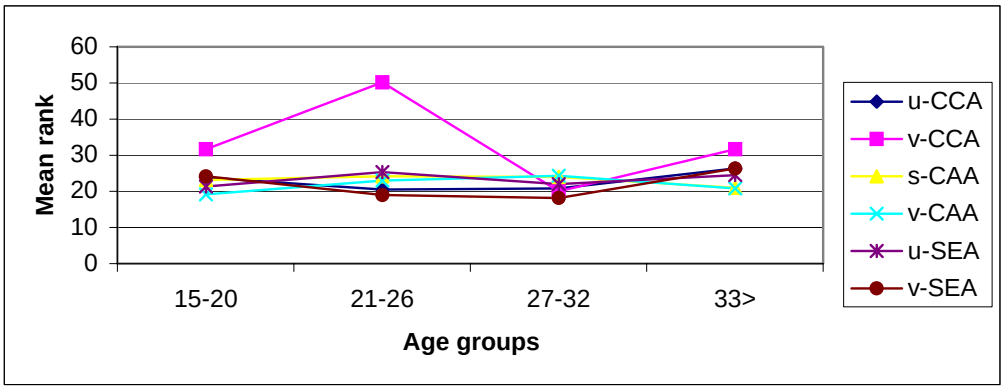


Figure (14): Age related mean rank of schistosome antigen levels measured in urine, serum and vaginal wash samples from endemic controls.

4.6.5.3. Age-dependent antigen levels among controls from non-endemic area

In contrast to what has been shown in cases and endemic controls, vaginal wash CCA measured in non-endemic controls had the lowest mean rank compared to other antigens measured in other samples especially among the 15-20 and 21-26 age groups. urine SEA showed a higher mean rank in the 15-20 age group that decreased in older groups. Urine CCA, serum CAA, vaginal wash CAA and vaginal wash SEA were found to be relatively stable (Figure, 15).

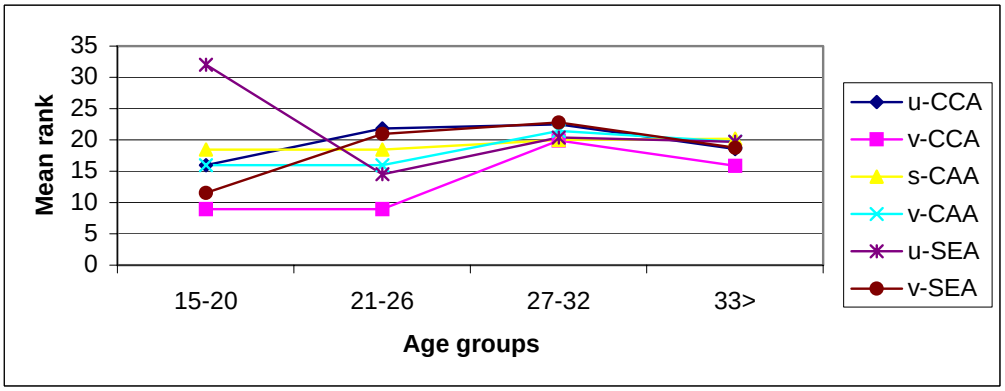


Figure (15): Age related mean rank of schistosome antigen levels measured in urine, serum and vaginal wash samples from non-endemic controls.

5. CHAPTER FIVE: DISCUSSION

5.1. Background

Morbidity related to Schistosomiasis haematobium in females' reproductive organs had been extensively discussed and different approaches were adopted in order to associate *S. haematobium* infection with morbidity in females upper and lower genital organs (Feldmeier *et al.,* 1992; Feldmeier *et al.,* 1993; Feldmeier and Krantz, 1993; Feldmeier *et al.,* 1994; Feldmeier *et al.,* 1995; Feldmeier, 1996; Gundersen *et al.,* 1996; Richter *et al.,* 1996; Helling-Giese *et al.,* 1996a; Helling-Giese *et al.,* 1996b; Kjetland *et al.,* 1996; Leutscher *et al.,* 1997; Feldmeier *et al.,* 1998; Vennervald *et al.,* 2000; Poggensee and Feldmeier, 2001). In Sudan, 4.85 million people are infected with schistosomiasis (18.16% of the whole population) and 24.03 millions are at risk (Chitsulo *et al.,* 2000). Just like many African countries endemic for schistosomiasis, Sudan lacks for a definitive information system about female genital schistosomiasis (FGS). However, clinicians working in North Kordofan and South Kordofan States, in the Midwestern Sudan, recognized granulomas in fallopian tubes and cervix of women infected with schistosomiasis. Spontaneously, clinician may decide to remove the granuloma surgically, a situation that could have been avoided if an earlier proper FGS diagnosis was performed. There is also the possibility of misdiagnosis and the risk of confusion with other tumors that would have negative implications to the patient. Morbidity in female genital organs has a wide range of causes; in which infections with parasites comes as one of the major factors leading to gynaecological problems especially in countries that lack proper control agenda for endemic diseases. The present study investigated morbidity in genital organs of females from *S. haematibium* endemic and non-endemic areas in Sudan; it also showed the possible role of immunoassays application for the diagnosis of FGS. Rahad area was studied for its

characteristic endemicity for Shistosomiasis haematobium for a considerably long time (>40 years). Identification of schistosome infection in endemic areas relies mainly on the microscopic detection of eggs in urine (*S. haematobium*) or stool (*S. mansoni*). Although species-specific egg detection is the gold standard technique for diagnosing schistosomiasis, high day-to-day fluctuation in egg excretion demanded the use of alternative methods. In 1989 Deelder and others highlighted the quantitative measurement of schistosome circulating antigens as a candidate alternative tool. Later on, such tests were used to quantify schistosome worm burden (Van Lieshout *et al.,* 1995; Van Lieshout *et al.,* 1998), detect active infections (Polman *et al.,* 1995; Van Lieshout *et al.,* 1997), and assess the effects of chemotherapy on schistosomiasis (Van Lieshout *et al.,* 1991; Van Lieshout *et al.,* 1993; Van Lieshout *et al.,* 1994; Guisse *et al.,* 1997). In contrast, results from serological tests for the detection of anti-schistosome specific antibodies are not well correlated with the worm burden as measured by the egg output. Another antigen that is associated to the miracidia trapped in the eggs, the soluble egg antigen (SEA), is also of a considerable value in the diagnosis of schistosomiasis especially the urinary form of the disease (Nibbeling *et al.,* 1998; Kahama, 1998). Schistosome circulating cathodic antigen (CCA) and circulating anodic antigen (CAA) appear to be the main genus-specific proteoglycan antigens. In 1989, Deelder and his colleagues developed an enzyme-linked immunosorbent assay (ELISA) test for the quantitative determination of CAA in serum. De Jonge *et al.,* (1990) followed by developing the same basic technique to quantify CCA in serum and urine. Both assays showed remarkably high sensitivities and specificities. Moreover, an assay was developed for the determination of SEA after several studies on antigen purification and quantification (Fu and Carter, 1990; Hassan *et al.,* 1992; Nourel Din *et al.,* 1994; Amanor *et al.,* 1996; Bosompem *et al.,* 1996). A monoclonal antibody based assay, to detect a 29 KD antigen that is species and stage specific, was developed for the diagnosis of *S. haematobium* infection and found to be effective in the field use (Bosompem *et al.,* 1996; Kahama, 1998).

5.2. A new trend for the understanding of schistosomiasis related morbidity

In the past all efforts concentrated on the treatment and control of schistosomiasis in the afflicted regions. Of course, some control programs achieved an appreciated success e.g. the Blue Nile Health Project (BNHP 1982- 1990) in Sudan but later faced problems that lead to many drawbacks, e.g. the increase of schistosomiasis prevalence after the termination of project. Morbidity is basically an individual concern event but in the case of infectious diseases it certainly has many impacts at the community level. The present aim of the global control strategy for schistosomiasis is to reduce its related morbidity in the population (WHO, 1985). Morbidity control calls for careful validation of direct and indirect markers of schistosomiasis morbidity, since monitoring the levels of morbidity has become as important as diagnosing infection (Vennervald *et al.,* 2000). Schistosomiasis haematobium is an important public health problem in Africa and the Middle East and the infection causes considerable morbidity in a high proportion of cases (Warren *et al.,* 1979). A potential area of application for a morbidity marker is genital schistosmiasis (Vennervald *et al.,* 2000). After many extensive studies, Poggensee and Feildmeier, (2001) focused attention on this clinical manifestation of schistosomiasis haematobium especially in females.

5.3. Assessment of genital morbidity due to *S. haematobium* infection in females

5.3.1. Ultrasonographic evidence: determination of abnormal echogenicity

Ultrasonography is an established method for detecting urinary tract pathology in the field (Hatz *et al.,* 1992). Ultrasonographic findings have shown to be well associated with standard direct and indirect measurements of schistosomiasis morbidity (Doehring *et al.,* 1985). In *S. haematobium* infections most of the morbidity is organ specific reflecting the localization of the adult worms and accumulation of eggs in the venous plexus around the urinary bladder and pelvic organs (Chen and Mott, 1989). Intensity and duration of exposure, different parasite strains, patients' age and genetic background are also factors that influence the evolution of pathology due to schistosomiasis. In S. *haematobium* infection, spontaneous healing after interruption of re-exposure may occur, but cases have been reported where urogenital lesions led to complications many years after exposure. (Richter, 2000).

In order to assess morbidity in females' pelvis that is associated with schistosomiasis in a Sudanese population, comparisons were made between cases passing *S. haematobium* eggs in urine and negative controls from the same endemic

area as well as controls from a non-endemic area. The risk of having abnormal echogenicity in general pelvis was found to be 3 times higher among cases infected with urinary schistosomiasis more than endemic controls; and approximately 8 times higher among cases more than non-endemic controls. Cases were also 9 times at risk of having abnormal echogenic findings in urinary tract and 6 times in urogenital tract more than endemic controls. Furthermore, *S. haematobium* positive cases were 3 times at risk of having genital tract abnormalities more than non-endemic controls. Cases were also at risk of having abnormal echogenicity ≈ 9 times higher in bladder and cervix more than endemic controls and ≈ 4 times higher in uterus more than non-endemic controls. The cervix was assumed to be the main target organ in genital schistosomiasis according to Kjetland *et al.,* (1996). Pathology in bladder, cervix and uterus were significantly prevalent among cases as compared to endemic and non-endemic controls. Sandy patches of the bladder associated only with females infected with *S. haematobium.* Calcification of the bladder and bulky cervix were also present among the cases as well as the endemic controls only.

5.3.2. Gynecological evidence

Clinically, intermenstrual or contact bleeding, especially postcoital, and dyspareunia may serve as diagnostic hints for vulvar, vaginal and cervical schistosomiasis (Gelfand, 1949). Delayed puberty, dysmenorrhoea and scanty menstrual blood flow are typical signs associated with ovarian schistosomiasis (Nabawy *et al.,* 1961). Vaginal discharge, dysmenorhea and pelvic pain are the most frequent gynecological complaints (Leutscher *et al.,* 1997). In the present study, it is worth mentioning that most of the gynecological symptoms were reported among the endemic controls who could also be considered as schistosomiasis exposed group. Cervical erosion, atrophied vagina, nodules in external genitalia, in addition to bulky uterus and swollen cervix were observed among cases infected with *S. haematobium* in urine. Polypoid lesions in cervix and vagina beside bulky uterus were reported among the non-endemic controls.

5.3.3. Possible role of indirect markers

In the case of *S. haematobium*, inspection for visible blood in urine or reagent strips for detection of microhaematuria have been used extensively, but it is not clear to what extent these results reflect significant urinary tract morbidity (Gryseels, 1989). Female physiology and anatomy facilitate mixing of urine samples with blood and vaginal secretions. For this reason, one thus has to question the assumption that haematuria, proteinuria and leukocyturia have the same diagnostic value in infected

women in different phases of life as in men (Gundersen *et al.,* 1996). Blood in urine was a common observation among females infected with *S. haematobium* while none of the controls experienced blood during urination. A significantly higher prevalence of microhaematuria was also reported among cases compared to endemic controls. The relationship between *S. haematobium* infection and body weight as well as hemoglobin level is still a matter of conjecture. Both cases and endemic controls had significantly lower body weights compared to non-endemic controls. Endemic controls had significantly lower hemoglobin levels compared to cases and non-endemic controls. Differences in both body weight and hemoglobin level could be explained by other confounders since both cases and endemic controls were exposed to infection and were having lower nutritional status related to economic status as compared to the non-endemic controls.

5.4. Diagnosis of female genital schistosomiasis

Traditionally, the diagnosis of genital schistosomiasis in women rely on biopsies which are invasive and not applicable in large-scale surveys and should only be performed when lesions that could have another cause are present in order to establish a definitive diagnosis (Vennervald *et al.,* 2000). In a pilot study conducted in rural Malawi, Kjetland *et al.* (1996) found that Colposcopy added some more information on the lesions observed by the naked eye but the smears obtained by a wooden spatula added little to the diagnosis. It was also found that the standardization of the procedure for vaginal lavage for measurement of ECP is mandatory since confounding factors like other genital infections and hormonal influences may exist (Witkin *et al.,* 1989; Belec *et al.,* 1995). Although the WHO, (2000b) workshop report divided pathological changes due to *S. haematobium* scored morbidity using ultrasound into those affecting the urinary bladder and those affecting the upper urinary tract, the measurement of genital tract morbidity was not mentioned.

5.4.1. What is the proper diagnostic tool?

A proper tool for the diagnosis of any infection is defined as a sensitive, specific, non-invasive, with reasonable cost, and an applicable technique at the community level. By definition, these conditions should also be applied when a technique is used for the diagnosis of female genital schistosomiasis. Until recently, the diagnoses of genital schistosomiasis in women relied on biopsies. In Tanzanian, based on biopsies examined under the microscope or histological sectioning from

females lower reproductive organs, 37% of the women had lesions where schistosome eggs were detected. It is worth noting that in some of these women eggs were not found in urine (Poggenensee *et al.,* 2000). A prevalence of 75% of vaginal schistosomiasis was reported by Renaud *et al.,* (1989); and 33% of cervical schistosomiasis was reported by Leutscher *et al.,* (1997). But still, Feldmeier *et al.,* (1993) doubted such an invasive procedure would be acceptable to women from different socio-cultural settings and its applicability in circumcised females. There is a need for an alternative method that is capable of over coming these difficulties but compromise has to be done in order to achieve the most possible benefits at the individual and community levels. Circulating antigen detection assays allowed a qualitative as well as quantitative detection of active schistosome infections (Qian and Deelder, 1982; Hayunga *et al.,* 1986; Feldmeier *et al.,* 1986). However, an improvement in the sensitivity is needed, especially for the diagnosis of schistosomiasis patients with light infection and for monitoring after chemotherapy (Van Lieshout *et al.,* 1992). Polman, (2000) showed the performance and practicability of circulating antigen detection assays in epidemiological studies and discussed their capacity and limitations. Specificity of antigen detection assays are considered to be high but evaluation has been based on samples tested from controls who have never been exposed to schistosome infection. It has been established that CAA or CCA detection has sensitivity at least equal to that of a 25 mg stool examination. False negative results occur in endemic areas. Antigen assays sensitivity and specificity largely depend on the intensity of infection of the study population, and on the parasitological examination as a reference method. Age is an important factor that may influence assays' performance. The fact that circulating antigen assays, like parasitological examination, miss a proportion of light infections has its consequences on epidemiological research especially with respect to its application in low endemic areas and after chemotherapy. False negatives by antigen detection and stool examination only partially overlap, which means that antigen levels are detected in some egg negative persons, and egg in antigen negative cases.

The detection of circulating antigens has shown to be effective to diagnose human schistosomiasis infections. Detecting the level of these antigens in cervico-vaginal secretions might be a promising approach for the determination of current infection in female genital organs. In addition, vaginal wash is an easy to perform and noninvasive procedure. The current study is the first to apply ELISA for the quantitative measurement of CCA, CAA together with SEA in genital fluids.

5.4.2. Diagnostic potential of circulating antigens in genital schistosomiasis

In order to test the diagnostic potential of schistosome circulating antigens detection, morbidity patterns discussed previously were related to schistosome antigen levels in samples collected from genital secretions as well as urine and serum samples. To start with, when testing the correlation of antigen levels with parasitological and indirect markers, serum CAA showed a positive correlation

with *S. haematobium* egg counts in urine. More over, urine SEA level was statistically higher among cases with positive microhaematuria tests than in negative ones (P= 0.04). High levels of CCA and SEA in cervico-vaginal secretions of females infected with *S. haematobium*; coincided with the presence of pathological manifestations in urogenital system scanned by ultrasound that differed significantly from control groups.

Measuring genital organs is a common investigation in basic gynecology. In the present study, ultrasound was used to measure width and length of uterus, and width of cervix in females infected with *S. haematobium* and non-infected females from endemic and non-endemic areas. An interesting finding was the CCA level in vaginal wash that negatively correlated with the uterus length. The same situation was for the SEA level in vaginal wash samples with uterus width and length. Furthermore, width of uterus decreased remarkably with the increase of SEA levels in urine samples and its length decreased with the increase of vaginal wash CAA. On the other hand, positive correlation between vaginal wash CAA and uterus length was found among the endemic controls and a slight decrease in cervix width correlated with the increase of CCA level in vaginal wash of the same controls. Obviously, antigen levels among cases showed significantly negative correlations with measurements of the uterus, this might be an indication of slight thickening in the examined organs that is correlated to antigen levels and thus to *S. haematobium* infection. It is rather difficult to prove such a theory at this stage, but this observation worth being noted and more investigations are needed.

Although there is no direct evidence that *S. haematobium* infection in females causes infertility, increased SEA levels in cervico-vaginal secretion and CAA levels in serum correlated with delayed puberty among cases. In addition, high levels of CCA in vaginal wash samples correlated with younger females and high levels of urine CCA correlated with diminished days of menstrual flow. Contrary to infected women, high levels of CAA in serum correlated with prolonged days of menstrual flow among controls from the endemic area. Moreover, CAA levels in serum and CCA levels in urine were higher among endemic controls who started menstruating earlier.

5.4.2.1. CCA, CAA and SEA assays specificities and sensitivities

In the present study, urine CCA specificity ranged from 91.1% to 94.9% and vaginal wash CCA specificity ranged from 83.7% to 94.9%. It was shown that CCA contains repeating units of lewis-X trisaccride, which may be cross-reactive with human inflammatory marker. These are expected to be more frequent in, and vary between, endemic populations (Polman, 2000). Lower CCA assay specificity in endemic areas is expected since exposure to infection is more frequent. Urine CCA assay reported a sensitivity ranging from 86.8% to 94.7% in a low endemic area in Burundi (Polman *et al.,* 1998). In the current study, urine CCA was not able to detect any of the urine parasitologically positive cases. Interestingly, CCA assay in vaginal wash samples, detected 16.7% of the urine positive cases.

Based on Previous studies, it was apparent that only the serum CAA detection could guarantee an invariably high specificity, irrespective of the target population (Polman, 2000). A specificity ranging from 91.1% to 92.3% was achieved by the serum CAA assay in females from Rahad area. Vaginal wash CAA showed lower specificities both in endemic and non-endemic areas ranging from 82.1% to 83.7%. Serum CAA assay previously had a sensitivity ranging from 65.1% to 69.8% in a low endemic area in Burundi (Polman *et al.,* 1998). In this study, serum CAA assay reported 31.3% sensitivity with reference to parasitological examination of urine samples. This result was combined by a lower sensitivity for the vaginal wash CAA assay (6.7%). The present study was conducted only in females at the childbearing age with generally low intensities of infection. Sex and age factors may certainly influence the sensitivity that could be achieved by the intended assays.

Urine SEA assay was 100% specific when tested in *S. haematobium* endemic and non-endemic settlements. Moreover, vaginal wash SEA assay was 100% specific in endemic area and 97.4% in non-endemic area. Although highly specific, the evaluation of the assay's performance has to be combined with its sensitivity especially in the case of genital schistosomiasis testing. Urine SEA assay was 21.2% sensitive in detecting cases passing *S. haematobium* eggs in urine. Ten percent (10%) of those cases were positive vaginal wash SEA assay. Previously, urine SEA assay using 2E6 monoclonal antibody reported 10% sensitivity in *S. mansoni* infected cases but none of the 47 individuals infected with *S. haematobium* gave positive reaction (Nibbelling *et al.,* 1998). Later, urine SEA assay sensitivity was improved and ranged from 76.2% to 78.8% (Kahama *et al.,* 1998b).

5.4.2.2. Correspondence of the assays' lower detection limits to egg count in urine

Deelder *et al.,* (1989) reported a lower detection level of <1 ng antigen/ml of CAA in serum that corresponded to a level of about 10 *S. mansoni* eggs/gm feces. In the current study, which investigated only urinary schistosomiasis, the lowest detection level of CAA in serum (5 ng AT/ml) corresponded to 4 eggs/10ml urine; 1 ng AT/ml vaginal wash CAA corresponded to 1 egg/10ml urine; 1 ng AT/ml urine CCA corresponded to 4 eggs/10ml urine; 3 ng AT/ml vaginal wash CCA corresponded to 4 eggs/10ml urine; 1 ng /ml urine SEA corresponded to 2 eggs/ 10ml

urine and similarly, 1 ng /ml vaginal wash SEA corresponded to 2 eggs/10ml urine. There are differences in worm fecundity between *S. haematobium* and *S. mansoni* that may explain the differences in the lower detection levels of schistosome antigens corresponding to the egg out-put.

5.5. A new tool for the diagnosis of female genital schistosomiasis

Assuming that all females passing eggs in urine are having genital involvement of schistosomiasis might be an overestimation. Assuming that the 16.7% vaginal wash CCA positives are cases of genital schistosomiasis is rather more reasonable especially when having clinical and gynecological signs. A reference method for genital schistosomiasis (e. g. biopsies) would have been of a value, but the invasiveness of such methods was an important factor that hampered the use of any. The high levels of vaginal wash SEA in endemic and non-endemic controls, although not positive, interrogated the value of vaginal wash SEA determination for the diagnosis of genital schistosomiasis. The use of CAA determination in vaginal wash samples for the diagnosis of genital schistosomiasis would not be recommended. But more investigations are needed to investigate the lower specificity especially in non-endemic area where the possibility of cross-reactive agents is raised.

Based on the present results, females positive for CCA level in vaginal wash samples were considered as cases positive for genital schistosomiasis. Accordingly the study groups were re-divided into cases positive for female genital schistosomiasis (FGS), cases positive for only urinary schistosomiasis, endemic controls and non-endemic controls. In total, 14 out of all groups were positive for genital schistosomiasis of which: 5 were passing *S. haematobium* eggs in urine, 7 from endemic controls and 2 from non endemic controls. The controls from endemic area who gave positive CCA tests in vaginal wash samples were considered as FGS cases with no egg excretion in urine. Although controls selection was carefully performed, 2 non-endemic controls were vaginal wash CCA reactive suggesting the possibility of having another cross reactive element.

As expected, high significant difference in vaginal wash CCA levels occurred between FGS cases compared to all other groups. Significant difference in vaginal wash SEA levels where also present among FGS cases compared to the rest of the groups (Tables, 12 and 13). Although SEA test in vaginal wash was not able to

identify genital schistosomiasis, it can be used as a confirmatory test after cases are defined by the CCA test in vaginal wash samples.

Table (12): CCA, CAA and SEA levels in females infected with genital schistosomiasis, urinary schistosomiasis, endemic controls and nonendemic controls.

		u-CCA	v-CCA	s-CAA	v-CAA	u-SEA	v-SEA
FGS	***Range***	100	1211	57	5	116	32
	MR	66.54	105.5	62.8	57.82	57.0	82.04
	N	14	14	14	14	14	14
Urinary schistosomiasis	***Range***	64	77	67	7	1641	79
	MR	52.7	52.3	64.7	52.0	71.4	57
	N	28	25	27	25	28	25
Endemic controls	***Range***	752	75	294	5	10	22
	MR	58.4	56.9	56.5	59.0	54.2	49.4
	N	38	36	38	36	38	36
Non-endemic controls	***Range***	820	70	59	7	5	33
	MR	61.5	40.4	54.5	56.7	55.3	53.4
	N	37	37	37	37	37	37
Sig.		0.6	0.000**	0.2	0.7	0.05	0.004**

Sig.: significance of difference
** difference is highly significant (P<0.01).
MR: mean rank
N: number of subjects.

Table (13): Differences in schistosome antigen levels, expressed as P-values, between cases of genital schistosomiasis with urinary schistosomiasis cases, endemic and non-endemic controls.

	u-CCA	v-CCA	s-CAA	v-CAA	u-SEA	v-SEA
FGS/ Urinary schistosomiasis	0.3	0.000**	0.8	0.4	0.2	0.02*
FGS/endemic controls	0.5	0.000**	0.1	0.9	0.8	0.001**
FGS/non-endemic controls	0.6	0.000**	0.2	0.9	0.9	0.002**

* difference is significant (P<0.05) ** difference is highly significant (P<0.01)

According to Kjetland and his colleagues (1996), there was no significant difference in the number of ova in the crushed cervical tissue of women with FGS compared to the women who were passing eggs in urine. On the other hand the present results showed that egg excretion in FGS cases was very few (Geometric mean: 2.0; Number: 14) compared to cases with only urinary schistosomiasis (Geometric mean: 6.2; Number: 29) and the difference was highly significant (P= 0.00). It is now possible to assume that FGS progression coincides with the decrease of egg excretion in urine but not necessary with egg count in genital tissues if the later was performed.

5.6. Progression of urinary schistosomiasis into genital schistosomiasis

In this study, when the CCA level was considerably high in vaginal wash samples, it gave low test results in urine; and when the levels of serum CAA and urine SEA were high, they showed low antigen levels in vaginal wash samples. This might be explained by the assumption that when the case is having active urinary schistosomiasis it develops high levels of antigens in urine and serum with low levels in genital secretion since infection of genital organs is not established yet. But when the female has higher levels of antigen in genital fluids, e.g. vaginal wash CCA, she tends to get low antigen levels in her urine presumably because she developed the genital form of schistosomiasis. This was supported by the observation of Poggensee *et al.,* (2000) that some of the women with genital schistosomiasis do not excrete eggs in urine.

Although no direct correlation was found between vaginal wash CCA levels and eggs out put, it correlated with serum CAA that had a significant correlation with eggs excretion. SEA levels showed no correlation with other antigens neither in urine nor in vaginal wash samples. A study conducted by Kahama *et al.,* (1998a) reported significant correlation between SEA and circulating anodic and cathodic antigens (CCA and CAA) in urine. Endemicity of the disease (low, moderate and high), intensity of infection, sex and age of the group under study and assay conditions might explain such antithesis since Kahama's study involved school children of both

sexes with high parasitemias; while this study included only females at the childbearing age with low intensities of infection. The possibility of false negatives by urine examination is more likely in endemic areas since controls are also exposed to infection.

5.7. Female genital schistosomiasis (FGS) and age factor

People living in an endemic area encounter infection early in their life. There is a slight increase in prevalence and intensity with age reaching a maximum in the 10 to 20 years of age. In the subsequent decades of life there is a drop in the prevalence accompanied by a drop in intensity (Butterworth *et al.,* 1988). In an age profile described by Kjetland *et al.,* (1996), who investigated females at the childbearing age, it was obvious that within age groups, schistosome ova detected per mm2 cervical tissue coincided with the decrease in ova per 10 ml urine and vise versa. As discussed previously, one may assume that egg excretion in urine decreased in those who developed genital schistosomiasis, usually elder ones.

Vaginal wash CCA level showed a typical contra verse pattern between cases and endemic controls in different age groups. When CCA level in vaginal wash of an age group increased among cases it decreased among endemic controls of the same age group and vise versa. On the other hand, Butterworth *et al.,* (1988) suggested an age-dependent acquired resistance to infection and its heterogeneous expression in different individuals that influence the age specific distribution of intensities of infection and of re-infection after treatment among communities living in endemic areas. More extensively, Fulford *et al.,* (1998) discussed other factors related to age and puberty, mainly hormones and immunity acquired over the years, speculating their effects on susceptibility to schistosome infection. The present study agrees with the previous findings in that

a drop in intensity of infection occurs in relation to increase in age, but it may be meaningful to highlight the assumption that the parasite explore and reside other organs over the years of active infection and it becomes no more feasible to trace it by urine examination.

5.8. Gender and cultural perspectives in schistosomiasis

Gender, as described by Feldmeier *et al.,* (1992) is the socio-cultural aspect of the male-female dichotomy and a concept overriding sex, which is considered to be restricted to the physiological biological aspect. The human physiology and disease biology, which we all share in common, does not give meaning to the symptoms that we can all understand. Each symbolic system develops according to its own rules and cultural environments add their difference. Social structure adds a further range of variation. So on top of, and interwoven with the sexual difference in disease manifestation between women and men, gender differences are determined by cultural and social factors that in their turn vary between different communities. Biased results in the assessment of pathology will lead to false estimates on the presence and degree of morbidity in women and thereby trigger conclusions on health priorities that are unwarranted. If the prevalence of schistosomiasis in the female genital tract is not known and means for assessment of morbidity of this female-specific disease entity virtually do not exist, it is unlikely that this debilitating form of schistosomiasis will ever receive a high priority by health policy decision makers. The spectrum of genuine and associated pathology, the sensitivity and specificity of diagnostic methods, and the efficacy of treatment in both sexes, as well as the role played by males and females in the transmission of the infectious agent and in disease control has to be established.

Diagnosis is central to all aspects of schistosomiasis: decisions on individual and community treatment, estimation on prognosis and assessment of morbidity, determination of transmission potential, and evaluation of treatment and of control measures (Feldmeier *et al.,* 1992). Diagnosis is often regarded as a purely technical discipline, belonging to the domain of health professionals in general, and the laboratory expert in particular. But in another sense it could be looked upon as a kind of interface between the infected individual, the infected community and the health

care system, represented by different types of health care professionals or researchers. Cultural and social factors are important in giving diagnosis form and content and have, for reasons of validity, to be acknowledged especially in dealing with communities and in control programs (Feldmeier *et al.,* 1993). Since there is a general consensus that the goal of control is the reduction of morbidity rather than the elimination of schistosomiasis as a disease, diagnostic means aimed at assessment of morbidity at the community level had been especially scrutinized by Feldmeier *et al.,* (1992). The acceptability of a given diagnostic procedure for the individual (male or female) differs between communities and is largely determined by the cultural and educational background. The efficacy of a diagnostic tool will be influenced by socio-culturally determinant factors. In the current study, the diagnosis of FGS was based on antigen testing of cervico-vaginal secretions. Vaginal wash was an easy to perform and short timed procedure, completely non-invasive and well accepted by investigated females and their families. A synoptic inventory (e.g. genuine and associated pathology, diagnosis, transmission, treatment and control) enables researches of different disciplines to systematically identify gaps in knowledge of gender-related determinants of infection in an encompassing sense (Feldmeier and krantz, 1993). As a consequence, validation of diagnostic techniques should not only be carried out for pure technical grounds, but socio-cultural aspects should also be considered to prevent false conclusions from research data (Feldmeier *et al.,* 1992).

5.9. Integrated research towards a schistosomiasis vaccine

Over the past 30 years there has been a concerted effort to understand host immune responses to schistosomes, with the ultimate aim of producing a vaccine for human use. Mature schistosomes inhibit the blood stream, potentially the most hostile immunological environment, yet they are long-lived parasites. Schistosome worms have evolved highly effective mechanisms for evading the consequences of the cellular and humoral immune responses which they provoke. This ability to evade the immune system appears to be due to several unusual parasite adaptations occurring soon after infection is initiated (Wilson and Coulson, 1998). Results from the present study showed representation of schistosome antigens in cervico-vaginal secretions that coincided with low antigenic levels in the urine and serum samples. Although it might be rather dashing to suggest a new strategy of immune evasion by schistosomes since immune response associated elements were not demonstrated in our samples,

shifting localization from urinary tract to genital organs in case of *S. haematobium* that was noticed by the high antigen levels in cervico-vaginal secretions with low antigen levels in serum and urine samples is an observation that worth more investigation.

The belief that humans are capable of mounting a protective immune response against schistosomes and therefore the development of a vaccine is feasible, is mainly based on the assumption that in natural circumstances, after years of exposure, individuals acquire effective immunity (Capron, 1998; Bergquist and Colley, 1998; Wilson and Coulson, 1998; Hagan *et al.,* 1998). The most important epidemiological argument is the consistent decline of infection rates in adults in endemic communities, in spite of continued exposure to infection (Hagan *et al.,* 1998). Studies in Burundi, Senegal and Kenya, had shown that such patterns were as consistent in newly exposed communities, where they cannot be explained by slowly acquired immunity (Gryseels, 1994; Stelma *et al.,* 1993; Ouma *et al.,* 1998). The evidence for effective acquired immunity to schistosomes in humans is not entirely consistent with epidemiological and biological observations. If it exists, its relative contribution to resistance may be less important than, or linked to, other age-related factors such as behavioral, hormonal or physiological changes in puberty (Gryseels, 1994). The safety and efficacy of any vaccine must be scientifically established before the vaccine can be applied. More than for any other known vaccine, the methodological and ethical challenges for schistosomiasis are considerable (Hagan *et al.,* 1998). A long and careful safety-assessment period should be undertaken before going into large-scale application. Moreover, the assessment should not be limited to the usual healthy adult males, because susceptibility to infection and disease in schistosomiasis varies greatly with age and gender (Gryseels, 2000). Vaccines might accumulate large sterile worm loads that cannot be diagnosed microscopically, but can still cause systemic, immunopathological and ectopic pathology, or do unpredictable rebounds of egg production. Such reduction of diagnostic possibilities raises serious medical and ethical concerns (Gryseels, 2000).

Chemotherapy strongly reduces egg counts; the difference between vaccine and placebo patients will have to be based on the follow-up of reinfection, which is often slow and unpredictable. At follow-up, positive individuals must be given chemotherapy, complicating further vaccine evaluation. Furthermore, egg counts and antigen detection, the available outcome measures of vaccination, cannot distinguish

anti fecundity from protective effects (Gryseels and De Vlas, 1996; Van Lieshout *et al.,* 1995). At the community level, it is already difficult to show the differential impact of various treatment strategies or of additional tools (Gryseels, 1996). Therefore, the impact of the partial protection expected from current vaccine candidates will not be easy to demonstrate. The effect on morbidity will be particularly hard to show, as it is even more profoundly and lastingly affected by chemotherapy than are egg counts (Wagatsuma *et al,* 1999; Frenzel *et al.,* 1999). The occurrence of schistosomiasis invariably reflects a lack of safe water, sanitation, education and accessible health care. A vaccine can further reduce the already weak commitment to the basic human needs as the best and most durable prevention against schistosomiasis and many other diseases (Gryseels, 2000).

Conclusions

The use of monoclonal antibodies based enzyme-linked immunosorbent assays for the detection of schistosome circulating antigens is a valuable tool for the diagnosis of schistosomiasis and has been further used in many other applications (Polman *et al.,* 1995; Guisse *et al.,* 1997; Van Lieshout *et al.,* 1998; Polman *et al.,* 2000). Although more is needed concerning the standardization of assays' sensitivities and specificities, the detection of schistosome circulating antigens, especially CCA, has a promising potential to be used as a diagnostic method for female genital schistosomiasis (FGS). The measurement of SEA after the definition of an FGS case may be useful as a confirmatory test. Ultrasonography can be a useful tool for the assessment of morbidity due to genital schistosomiasis especially in the field conditions or when other diagnostic tools are not at hand. Gynecological examination and indirect markers can serve as hints, but not quite specific, for genital schistosomiasis. More samples and a designed study are required to obtain significant gynecological and obstetrical observations, which might be associated with *S. haematobium* infection in females.

The present study has shown the spacious prospects of circulating antigens determination in the field of schistosomiasis related morbidity assessment and diagnosis where a confirmatory test can be more evaluated. Immuno-assays can also be used to differentiate between only urinary and genital forms of schistosomiasis. It is worth mentioning that other reasons may be responsible for the drop in prevalence and intensity of infection correlated to age increase. More understanding of the host parasite relationship and schistosomiasis related morbidity is needed to support such an assumption. Finally though very important, many factors may influence the diagnosis of genital schistosomiasis in females (e. g. strain variation, false negatives

by urine examination, age, virginity, pregnancy, socio-cultural factors, etc.) and should be carefully considered in future studies.

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LIBYA
EGYPT
Ḥalāʾib
SAU. AR.
Wādī Ḥalfāʾ
Port Sudan
Red Sea
Nile
Sawākin
Atbara
CHAD
Omdurman
Khartoum
Kassala
ERIT.
Wad Madanī
Al Fāshir
Al Rahad
Al Qaḍārif
Al Ubayyiḍ
Kūstī
Nyala
White Nile
Blue Nile
Malakāl
ETHIOPIA
Wāw
C.A.R.
Bor
Juba
Nimule
Kinyeti
DEM. REP. OF THE CONGO
UGANDA
KENYA
0 300 km
0 300 mi
24
30
36
18
12
6

QUESTIONNAIRE SHEET
Morbidity assessment of *S. haematobium* infection in females
Tropical Medicine Research Institute, 2002

Date …….. Serial number: ……..……

Name of hospital ……..…… Hospital record (if available): ……..

Name …… Age ……

Residence ……..…… Occupation ……

What do you complain from? (Hospital report if available)

Menstrual disorders? Yes ( ) No ( )
Intermenstrual bleeding? Yes ( ) No ( )
Postcoital bleeding? Yes ( ) No ( )
Dyspareunia? Yes ( ) No ( )
Lower abdominal pain? Yes ( ) No ( )
Back ache? Yes ( ) No ( )
Vaginal Discharge? Yes ( ) No ( )
Pruritis? Yes ( ) No ( )

Other associated symptoms ……..

……..

Have you ever been infected with schistosomiasis before? Yes ( ) No ( )

Have you ever-noticed blood in urine? Yes ( ) No ( )

If any of the above is yes, how old were you at that time? ……..……..

<u>GYNECOLOGICAL HISTORY</u>

Menarche ……..
L.M.P. …… Kata ……
Period associated with clots Yes ( ) No ( )
Cycle regular ( ) irregular ( )
Dysmennorrhoea present ( ) Absent ( )
Contraception use present ( ) Absent ( ) Duration of use …….
Menopaused Yes ( ) Yes ( ) Age at Menopause ……....

OBSTETRICAL HISTORY

Gravida …… Para …… + ……
Age of youngest Sibling ……...
History of an Ectopic Pregnancy Yes ( ) No ( )
History of previous Abortions Yes ( ) No ( )

PAST MEDICAL/ SURGICAL HISTORY

Past History of any surgical intervention Yes ( ) No ( )
Past History of STD Yes ( ) No ( )

PHYSICAL EXAMIATION

Body weight: ……..……..……..……
Blood pressure: ……..……
Tender abdomen: ……...……..…..
Supra-pubic tenderness: ……
Splenomegaly: ……..…….

GYNECOLOGICAL EXAMINATION (EMPTY BLADDER)

External genitalia (Vulva & Clitoris)		**Comments**
Normal	( )	……..
Swollen	( )	……..
Ulcerated	( )	……..….
Nodulated	( )	……..
Hypertrophied	( )	……..……

Vaginal examination

Normal	( )	……..
Fibroid-like	( )	……..
Polypoid lesions	( )	……..

Cervix,

Normal	( )	….……...……..
Cauliflower-like	( )	……..
Nodular hypertrophy	( )	……..
Ulceration	( )	……..
Polypoid lesions	( )	……..
Sandy patches	( )	……..

Corpus of the uterus

Normal	( )	……..
Bulky	( )	……..

INVESTIGATIONS

HB level: ……

-Ve S.H.	+Ve S.H.		Haematuria	Leukocyturia	Proteinuria
	1st count				
	2nd count				

ULTRASONOGRAPHY (FULL BLADDER):

Organ	+ (cm)	X (cm)	Comments
Cervix			
Uterus			
Abnormal echogenicity			

Form of consent

Date: ……..

Name: …….

Signature: ……

I herby, name and signature above, agree to submit my self to all required investigations and to give samples during the course of the study entitled: Morbidity assessment and diagnosis of schistosomiasis haematobium in female reproductive organs in the Midwestern Sudan. I was informed that this study is approved by the autharized committee from the Ministry of Health. I was also insured that I will not be harmed by any mean and that treatement will be provided. I understand that treatment will not be interrupted even if I decided to withdraw at any time during the study.

Signature of investigator: …….

(Arabic translation: form of consent)

اقرار بالموافقة

التاريخ:.....

الاسم: .....

التوقيع: .....

أوافق أنا المذكور اسمي و توقيعي أعلاه على الخضوع للفحوصات و اعطاء العينات اللازمة للقائمين على إجراء البحث بعنوان: *الكشف عن المرضية المسببة من البلهارسيا البولية في المناسل الأنثوية بمنطقة وسط غرب السودان* و الذي تمت إجازته أخلاقيا من قبل اللجنة المختصة بوزارة الصحة الاتحادية .
على أن يتم ضمان عدم حدوث أذى و أن يتكفل فريق الدراسة بعلاج المرض موضوع الدراسة و أن يكون لي الحق بالانسحاب في أي وقت خلال الدراسة على أن لا يؤثر هذا على سير العلاج.

توقيع الباحث: .....

Preparation of buffers

0.035 M PBS pH 7.8 (general purpose)

5.7 g of $Na_2HPO_4 \cdot H_2O$ (MW 178)

0.48 g of KH_2PO_4 (MW 136)

8.5 g of NaCl (MW 58.4) in 1000 ml H_2O, check pH (should be 7.2-7.8)

(for preservation add 0.05% NaN_3)

CCA assay buffer, pH 7.8

5 g of PEG-1000 (or 50 ml of a 10% solution)

670 µl of 30% Tween-20 in distilled H_2O

in 1000 ml PBS (just before use add 0.1% BSA, high quality)

CCA assay neutralization buffer (final volume 25 ml)

1 M NaOH (1.22 ml)

1 M phosphate* (2.5 ml)

30% Tween-20 (0.3 ml)

5% BSA (1 ml)

25% PEG-1000 (1ml)

H_2O (19.2 ml)

CAA assay buffer

0.010 M phosphate buffer, pH 7.0

269 mg of $NaH_2PO_4 \cdot H_2O$ (MW 138)

545 mg of $Na_2HPO_4 \cdot H_2O$ (MW 178)

2.92 g of NaCl (MW 58.4)

215 mg $MgCl_2$

in 500 ml highly purified H_2O, check pH, should be 7.0 (for preservation add 0.05% NaN_3)

(just before use add 0.3% Tween-20 and 0.1% BSA (high quality))

CAA assay neutralization buffer (final volume 25 ml)

H_2O (18.8 ml)

1 M NaOH (1.22 ml)

1 M phosphate* (2.5 ml)

0.1 M $MgCl_2$ (1ml)

5% BSA (1ml)

30% Tween-20 (0.5 ml)

* 1 M phosphate is composed of about 3 parts of 1 M Na_2HPO_4 (of 50ºC) + about 1 part of 1 M KH_2PO_4 until pH=7.3

DEA buffer (for pNPP substrate 1 mg/ml)

1 M $MgCl_2$ (MW 95.22) in 10 ml distilled H_2O

Add 4.75 ml DEA (MW 105.14) and 250 µl of the 1 M $MgCl_2$ solution to 450 ml of H_2O, adjust the pH to 9.6 and add distilled H2O until a final volume of 500 ml

4% (w/v) TCA

Dissolve 4 g Trichloroacetic acid (MW 163.39) in 100 ml H_2O (0.24 M), store at 4ºC (dissolve a complete bottle of TCA until a concentrated TCA-solution, e.g. 70%, 250 g in 357 ml).

PNPP substrate (para-NitroPhenylPhosphate)

Dissolve 1 mg per ml of DEA buffer

0.1 M sodium carbonate buffer

0.795 g Na_2CO_3

1.465 g $NaHCO_3$

to 450 ml H_2O, adjust pH to 9.6 and add H_2O until a final volume of 500 ml, store at room temperature).

SEA assay buffer (make fresh)

Dilute 0.1% bovine serum albumine (BSA) (1 g/ml)

0.3% Tween-20 (3 µl/ml)

in 35 mM PBS