Detection of the circulating antigens CAA and CCA in human Schistosoma infections : immunodiagnostic and epidemiological applications

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Cover illustration: Egyptian hieroglyphic script depicting haematuria, probably due to schistosomiasis haematobium (based on the papyrus of Kahun, 1900 B.C.).

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Contents

List of abbreviations

AWA	adult worm antigen
AWA-TCA	trichloroacetic acid soluble fraction of AWA
BSA	bovine serum albumin
95%CI	95% confidence interval
CAA	circulating anodic antigen
CCA	circulating cathodic antigen
ELISA	enzyme-linked immunosorbent assay
epg	eggs per gram of faeces
e/10ml	eggs per 10 ml of urine
FITC	fluorescein isothiocyanate
IC	immune complexes
IFA	immunofluorescence assay
mg/kg	milligram per kilogram (body weight)
MAb	monoclonal antibody
n	number
PBS	phosphate-buffered saline
SD	standard deviation
SEA	soluble egg antigen
TR-IFMA	time resolved immunofluorometric assay
v/v	volume/volume
w/v	weight/volume

Preface

Schistosomiasis (bilharzia) is one of the major parasitic infections in tropical areas. It is caused by blood-dwelling flukes, residing in the mesenteric and pelvic veins of the human host. Over 200 million individuals are estimated to be infected with these worms, while at least 700 million people are at risk.

The conventional method to diagnose this disease is by the demonstration of parasite eggs in faecal or urine specimens. However, this technique has several disadvantages, a.o. infections with low worm burden can be easily missed, and due to a high day-to-day fluctuation in egg counts, repeated examinations are needed to estimate the intensity of infection. Alternatively, schistosomiasis can be diagnosed by the detection of host antibodies directed against schistosome antigens. This technique has shown to be highly sensitive and specific, but is not able to differentiate between active and past infection, or to give information about intensity of infection.

A comparatively new approach for the diagnosis of schistosomiasis is the demonstration of *Schistosoma* antigens in the circulatory system or the urine of the host. Although these so called "circulating antigens" may be produced by several parasite life cycle stages, most research has been done on two glycoconjugates which are associated with the gut of the adult worm, namely circulating anodic antigen (CAA) and circulating cathodic antigen (CCA).

Initial research (performed in our laboratory) on the diagnostic applications of these antigens for human schistosomiasis was mainly focused on the demonstration of CAA in serum and urine. These studies, compiled in the thesis of Dr. N. de Jonge (1990), showed the levels of CAA in serum and urine to be correlated with the intensity of infection, as determined by egg output. In addition, following successful treatment of the parasite infection, CAA levels in serum were found to decline rapidly.

The research described in this thesis should be considered as a continuation of the initial work performed by De Jonge *et al.*. It covers a period of seven years, of which approximately the first three years have been spent at the NAMRU-3 in Cairo, Egypt, the other four at the Department of Parasitology of the University of Leiden, The Netherlands. The main objectives of this study were to further elaborate the assessment of CAA and CCA concentrations in human serum and urine samples, in particular for the diagnosis of an active *Schistosoma* infection and the assessment of cure following chemotherapy. In addition, as serum and urine levels of CAA and CCA were found to be correlated to the number of worms present, we also evaluated the use of antigen determination in more epidemiologically-oriented studies, that is to say to examine infection patterns and the dynamics of worm populations in humans living in endemic areas.

Chapter 1 of this thesis includes a general introduction to human schistosomiasis, with particular emphasis on the diagnosis of this parasitic disease. In addition, a literature overview is presented dealing with the characteristics of CAA and CCA, and with the determination of CAA and CCA in serum and urine, both in experimentally infected animals and in humans.

Chapter 2 describes the experimental work, the majority of which has also appeared as papers in scientific journals. These publications are included as such without any modifications, which has resulted in some overlap, particularly in the "introduction" and "material and method" sections. Furthermore, some inconsistencies between the different papers were unavoidable due to the fact that a few technical aspects of the circulating antigen assays have changed during the years in which this work was performed.

A comparative evaluation of CAA and CCA determination in serum and urine samples, collected from one and the same group of schistosomiasis patients, is reported in **chapter 2.1**. Improved diagnostic performance was achieved by parallel testing and combining the results of the individual assays. Combined measurement of CAA and CCA in one assay resulted in a lower sensitivity, as described in **chapter 2.2**. The kinetics of CAA and CCA in serum and urine following chemotherapy, are described in **chapters 2.3** and **2.4**, while in **chapter 2.5** the determination of CAA and CCA was evaluated as a tool to compare the efficacy of different treatment regimens. The diagnostic performance of CAA and CCA determination in humans with low and recent *Schistosoma* infections, is described in **chapters 2.6** and **2.7**, respectively. A more simple technique to pretreat serum and urine samples is reported in **chapter 2.8**. In addition, this

chapter contains a further evaluation of the sensitivity and specificity of the CAA and CCA assays, both for serum and urine. The use of serum CAA and CCA determination for the assessment of worm burdens in humans is reported in **chapters 2.9** and **2.10**. Antigen levels are analyzed in relation to the age of the host, the parasite egg counts and the transmission level of the endemic area.

Chapter 3 contains a general discussion of the work performed in this thesis. The technical aspects of circulating antigen determination, like sensitivity, specificity and diagnostic performance of the employed assays, as well as the collection of material and the practical applications of circulating antigen determination, have been extensively discussed in the thesis of De Jonge. Therefore, this chapter puts more emphasis on the comparison between parasitological examination and the determination of circulating antigen levels, both as a tool for the diagnosis of schistosomiasis, as well as for the assessment of worm burden in more epidemiologically oriented studies. In addition, this chapter contains a short literature overview of other circulating antigens, apart from CAA and CCA, which have been described for the diagnosis of an active *Schistosoma* infection in humans.

Chapter 1

General introduction

Human schistosomiasis

Schistosomes belong to the class of Trematoda (Platyhelminthes). There are five schistosome species which can be parasitic to man: *Schistosoma mansoni*, *S. japonicum*, *S. haematobium*, *S. intercalatum* and *S. mekongi*, of which the first three are the most common. These species differ in a number of important ways, a.o. the species of the (intermediate) snail host, the final location in the (definitive) human host, the numbers of ova produced, the size and shape of the eggs and the pathology they induce.

Figure 1 shows an adult male and female schistosome worm. The male is 1 to 2 cm long and embraces the thinner and longer female in a groove on its ventral side called the gynaecophoric canal. The adult schistosome worms have an average life span of 2 to 5 years, but some may live for up to 40 years.[1-6]

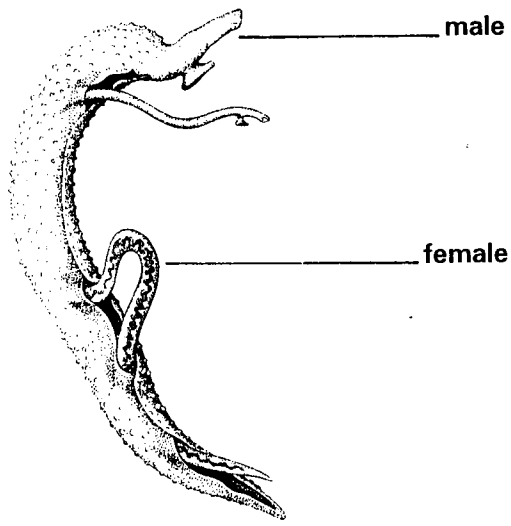

Figure 1. Paired adult *Schistosoma* worms.
From Gönnert, R. (1948). Die Struktur des Korperoberfläche von *Bilharzia mansoni* (Sambon, 1907). Zeitschrift für Tropenmedizin und Parasitologie, **1**, 105-112. Reproduced with kind permission from Georg Thieme Verlag, Stuttgart.

The digestive system comprises a short oesophagus and an intestine which divides into two lateral gut caeca. The worms feed on serum globulins, haemoglobin and other components of the host blood. As schistosomes have no anus, undigested material, including parasite antigens, is excreted by regular regurgitating.

Figure 2 illustrates the transmission cycle. Adult worms do not multiply in the human body. The ova produced by the worms need to leave the body to continue the life cycle and transmission has to take place via the appropriate intermediate snail host. The number of eggs daily produced by a mature female worm varies from several hundreds to several thousands, depending on the species. Histolytic enzymes, secreted by the next larval stage called miracidium, enable the eggs to penetrate through the host tissue to bladder (*S. haematobium*) or intestine (other species), where they are excreted with the urine (*S. haematobium*) or faeces (other species). About half of the number of eggs, however, do not reach the intestinal or vesical lumen. They are carried away with the blood stream and/or trapped in the tissues. These retained eggs provoke a granulomatous inflammatory response; they are the main cause of the pathology induced by the parasitic infection.

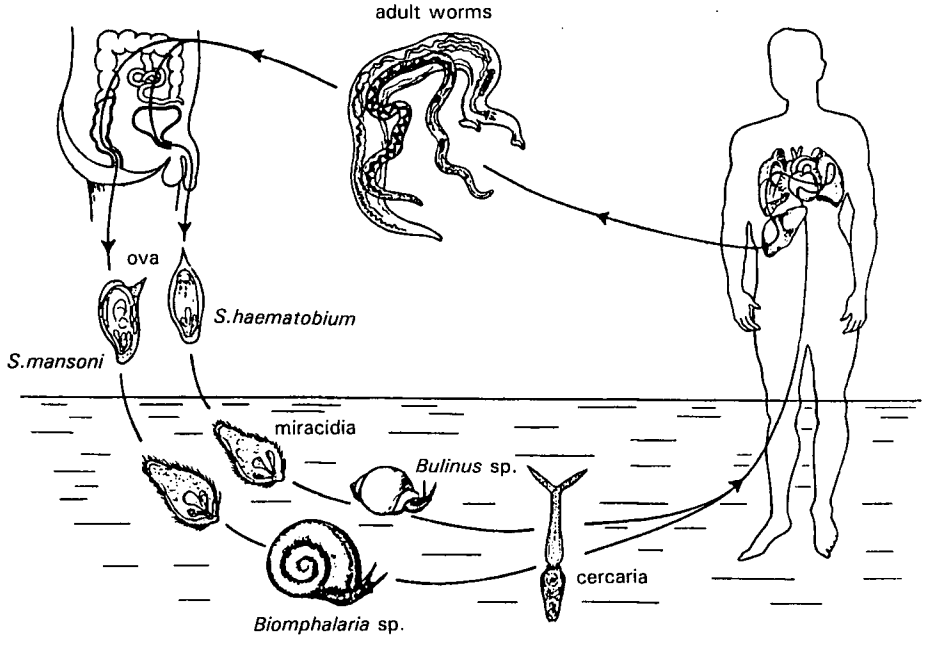

Figure 2. Life cycle of *Schistosoma mansoni* and *Schistosoma haematobium*. (Courtesy of Dr. A.M. Deelder)

When an excreted egg reaches fresh water, it hatches and the miracidium emerges. This larva must, to develop further, penetrate a compatible fresh water snail within a period of several hours. The snails (Gastropoda) susceptible to *S. mansoni* belong to the genus *Biomphalaria*, for *S. haematobium* and *S. intercalatum* to the genus of *Bulinus*, while *S. japonicum* and *S. mekongi* are transmitted by the genus *Oncomelania* and *Neotricula*, respectively. In the snail, massive asexual multiplication occurs and several generations of multiplying larvae (sporocysts) develop. Eventually, these sporocysts produce large numbers of infective larvae (cercariae), which leave the intermediate host in numbers up to 3000 cercariae per snail per day.

When a human host is exposed to infested water, cercariae are able to penetrate the skin within minutes. Via the blood stream, the young parasites (schistosomula) migrate, in a period of a few weeks, via the lungs to the liver where the adult worms pair. The paired worms migrate to the pelvic (*S. haematobium*) or the mesenteric (other species) veins and about 4-7 weeks after infection egg production starts. The number of adult worms present in an infected person is estimated to range from a couple of dozens, up to several thousands.[6]

Occasionally, severe itching and dermatitis may develop shortly after cercarial penetration of the skin, particulary in those individuals who are exposed for the first time. These symptoms; however, are usually mild and often pass unnoticed. Severe dermatitis may also result from invasion of non-human schistosome cercariae, giving rise to 'swimmers itch', which is common in some non-tropical environments.

Acute schistosomiasis or 'Katayama fever' is associated with the maturation of the worms and the onset of egg production, and usually occurs 3 to 9 weeks after infection. Symptoms are often non-specific, not related to the intensity of infection, and more often noticed in individuals infected for the first time. *Schistosoma* infection can be very difficult to diagnose at this stage, also because it is usually to early to demonstrate eggs in the excreta.[7,8]

As mentioned above, the disease of schistosomiasis is mainly due to the formation of granulomata around the parasite eggs which are trapped in the host tissue. Subsequently, this granulomatous inflammatory response may lead to fibrosis of the liver or urinary bladder, depending on the species. Initial symptoms of chronic schistosomiasis may be diarrhoea, dysenteria, abdominal pain, lack of appetite, loss of weight, bloody stool, haematuria, proteinuria, while severe infections may result in portal hypertension, hepatomegaly, splenomegaly, ascites, bleeding varices in intestinal schistosomiasis, or obstruction of the urinary tract in urinary schistosomiasis. Mainly based on

epidemiological data, schistosomiasis has also been associated with the incidence of several types of cancer, of which the correlation between *S. haematobium* infection and cancer of the bladder seems to be most apparent.[9,10]

The mortality rate due to schistosomiasis is not high, and even serious hepatic or urinary morbidity develops only in a small percentage of those infected. Still, high prevalences of less severe morbidity (e.g. bloody diarrhoea or haematuria) could have a significant impact on the general health status of the involved communities.[11]

Intensity and duration of infection have been pointed out by many investigators as the major factors determining development of serious chronic diseases in infected individuals, but has been disputed by others.[12] Probably also other factors (*a.o.* genetic and immunological background) play an important role. When comparing different communities, the relation between intensity of infection in a certain population and the prevalence of morbidity seems to be more pronounced.[12]

The geographical distribution of schistosomiasis is both linked to the distribution of the intermediate hosts, which breed only in tropical climates, and the lack of proper sanitation and water supply. Although effective drugs are available, the number of areas where transmission is taking place is still increasing, due to changing ecological and socio-geographical factors (e.g. irrigation projects; human migration). Schistosomiasis is endemic in approximately 75 countries in the tropics, reaching from China, via the Middle East and Africa, to Brazil.[13,14]

The dispersion of schistosomes in the human host population follows a negative binomial distribution, i.e. in endemic areas the majority of the parasites is present in a small fraction of the infected individuals. In addition, there is a characteristic age-related prevalence and intensity of infection, with a pronounced peak in egg excretion during adolescence, which rapidly decreases in adults. Figure 3 shows an example of such a typical age-intensity curve for a human population living in an area with high *S. mansoni* transmission.

The introduction of relatively safe and effective chemotherapeutic treatment has improved the control of severe schistosomiasis. Although frequent mass treatment may reduce the morbidity of the target population,[15] some reports have shown little or no effect.[16,17] The available drugs, however, are not preventive. In endemic areas, therefore, a large proportion of the population requires regular retreatment because of continuous re-exposure. Besides, control programs based only on treatment seem to have no effect on the transmission.[18] In addition, regular treatment programs are costly and difficult to sustain. These

facts provide a justification for vaccine development research.

Of the several available schistosomicides, praziquantel is nowadays considered as the drug of choice. It is most widely used as either Biltricide®, produced by Bayer Leverkusen, Germany, or Distocide®, produced by Shin Poong Pharmaceutical Co. Ltd, Korea. The standard advised dosage is a single oral dose of 40 mg per kilogram of bodyweight (kgbw) for all species, except for *S. japonicum* and *S. mekongi*, where the current regimes are either three doses each of 20 mg/kgbw or two doses each of 30 mg/kgbw, all given in one day.

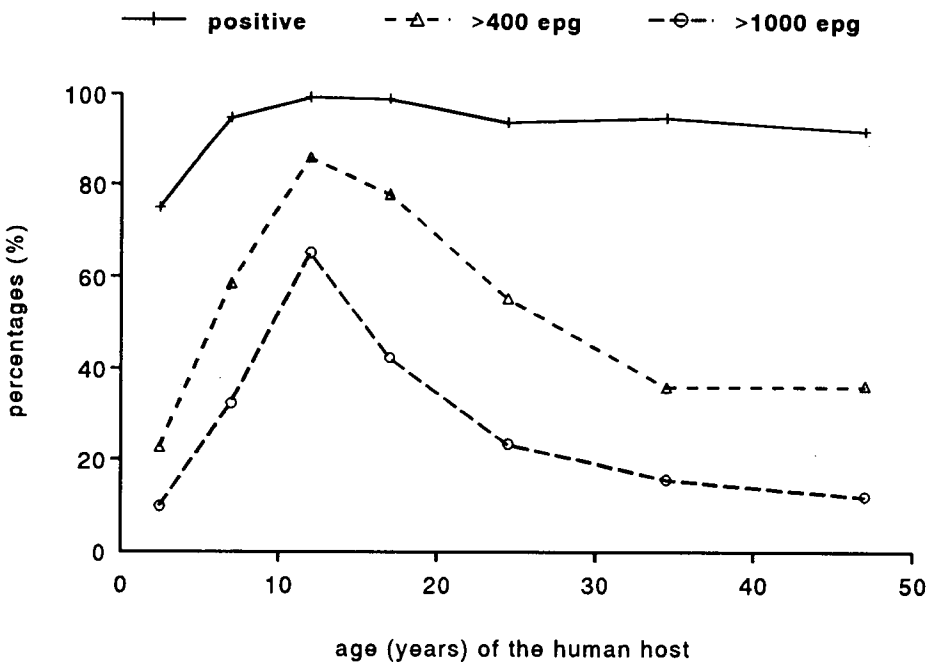

Figure 3. Example of an age-prevalence curve for a human population living in an area with intensive *S. mansoni* transmission (n = 1185). epg = *Schistosoma* eggs per gram faeces. Data from Tshamaka, Maniema, Zaire, with kind permission of Dr. A.M. Polderman.

Diagnosis of schistosomiasis

Evidently, accurate and sensitive diagnosis of schistosomiasis is of great importance, both at individual and community level. A diagnostic procedure can be used for a number of applications, ranging from clinical diagnosis of an individual case to the evaluation of control measurements. In addition,

diagnostic assays can also serve as a research tool, for example in epidemiological studies, or for the evaluation of possible vaccine candidates. This central role of diagnosis has been recently extensively reviewed by Feldmeier and Poggensee.[19]

The ideal diagnostic procedure should have both a high specificity and sensitivity, so it could be used to diagnose light infections and to assess failure of treatment. It should be kept in mind that the predictive value of a diagnostic test is not only related to specificity and sensitivity of the assay concerned, but is also depending on the prevalence of infection in the studied population.

Ideally, the diagnostic method of choice provides both qualitative and quantitative information, as the development of disease may be linked with intensity of infection. In addition, the ideal test should be able to diagnose the disease early after exposure. Furthermore, the procedure should be easy to perform, also under field conditions, and preferably without the need of skilled personnel or expensive equipment. Also, test results should be reproducible and simple to interpret.

Until now no single diagnostic test for schistosomiasis is available which fulfils all these requirements and the methods which are available range largely in their characteristics. Therefore, the decision which of the current methods to use depends on several factors, a.o. the logistic situation (e.g. whether it is applied for a detailed study performed in a well equipped laboratory or for the clinical diagnosis of individuals performed under field conditions) and the type of information needed (e.g. the presence of an active infection, the state of infection, the pathology induced).

The currently available procedures for the diagnosis of schistosomiasis can be generally divided in three different approaches: (i) direct parasitological methods, (ii) indirect methods and (iii) immunological methods, which can be either direct or indirect.

Parasitological methods

The most widely used diagnostic method is the direct demonstration of parasite eggs after microscopic examination of stool or urine. *Schistosoma* eggs are not difficult to identify. They are 70 to 180 μm long and feature a typical terminal (*S. haematobium, S. intercalatum*) or lateral (other species) spine.

In *S. haematobium* infections, eggs can be detected in a urine sample following sedimentation, centrifugation, or filtration. The technique which is nowadays most commonly used in epidemiological studies is the filtration of 10 ml of urine with a syringe over a nylon or paper filter. If such a quantitative diagnosis has been performed, intensities of infections are usually expressed as eggs per 10 ml of urine (e/10ml).

In the other *Schistosoma* species, eggs can be demonstrated in a direct faecal smear. Most widely used in epidemiological settings is the Kato-Katz method, in which a standard amount (generally a volume equivalent to 25 mg of faeces) is first sieved to remove coarse particles and then cleared by the addition of glycerin. Although this technique is based on volume and not on weight, results are usually expressed as eggs per gram faeces (epg). Concentration techniques, such as the formol-ether or glycerine sedimentation method, usually increase the sensitivity, but give only semiquantitative information and are more difficult to perform.[20]

Hatching methods can be used for qualitative diagnosis and to check the viability of eggs. The detection of viable eggs may be of importance for the assessment of cure, as dead eggs can be present in the excreta for several weeks after chemotherapy, particularly in *S. haematobium* infections. Although sensitive, this technique can have variable test results. Examination of rectal biopsy specimens are also used for the qualitative diagnosis of *Schistosoma* infections. This is, however, an invasive technique not acceptable for routine screening.

In principle, parasitological methods have a specificity of 100%, although in practice mistakes may be made. The sensitivity, however, can be low, particularly in *S. mansoni* infections where the amount of examined faeces in a direct faecal smear is relatively small in relation to egg excretion. In *S. japonicum* infection egg production is much higher, but there the distribution of the eggs in the stool can be very irregular. So in either case, light infections can be easily missed. Besides, a strong day-to-day fluctuation has been described. In order to pick up light infections and to obtain a more accurate measure of the intensity of infection, repeated examinations are necessary.[21-24] Even so, in very light infections, the number of excreted eggs is often so small that even after repeated microscopical examination, the diagnosis cannot be confirmed.[7,8,23] The shortcomings of the parasitological methods will be more extensively discussed in chapter 3 (general discussion) of this thesis.

Indirect methods

Schistosomiasis can also be diagnosed by indirect methods, based on the use of clinical, biochemical or immunological disease markers. These techniques are more related with the presence of pathology than with the actual infection and therefore will only be very briefly discussed in this paragraph. For a more detailed review, see Feldmeier and Poggensee.[19]

The clinical diagnosis of schistosomiasis can be very difficult, as most symptoms are non-specific and easy to confuse with those of other diseases.

Only in chronic urinary schistosomiasis the clinical signs of infection are more specific. The distribution of questionnaires was found to be useful for the identification of areas of high risk for urinary schistosomiasis.[25] Also haematuria, proteinuria and dysuria seem to be useful indicators for the presence of a chronic *S. haematobium* infection. A dip-stick method to determine haematuria has been shown to be a sensitive technique, suitable for mass screening, although with a low specificity.[26] Recent studies indicated eosinophil cationic protein (ECP) in urine as a potential marker for *S. haematobium* induced morbidity.[27,28]

Due to its technical improvement and field-applicability, ultrasonography is increasingly used, both in hospitals and at the level of field studies, allowing visualization of the pathological anatomical changes caused by the *Schistosoma* infection.[29]

Immunological methods

Immunodiagnostic techniques can be generally divided in three categories: antibody detection, measurement of circulating antigens, and other techniques, e.g. the cellular immune response to certain schistosome antigens. Here, only the first two will be discussed. A list of review papers, some of which contain a detailed description of the different immunological techniques, is given at the end of this chapter.

A wide range of tests is described for the demonstration of specific antibodies, including different assay formats, the use of crude, purified or recombinant antigens and the detection of specific antibody isotypes. Although most of these techniques have shown to be highly sensitive and specific, their value is considerably lowered by the fact that antibody levels generally do not differentiate between present and past infection and do not give any information about the intensity of infection. The use of purified antigens, such as for example the egg antigen CEF6, seems to give somewhat better results.[30] However, the preparation of purified antigens still demands large quantities of parasite material. Until now no generally applied antigen has become available via recombinant DNA technology.

For these reasons, the application of specific antibody detection is only suitable in specific situations. It is, for example, a sensitive method to diagnose individuals originating from non-endemic areas who have been exposed incidentally.[7,31] It also can be used as a tool for epidemiological studies in areas where the prevalence of schistosomiasis is so low that the classic parasitological methods fail.

Table 1. Characteristics of CAA and CCA.[1]

Characteristics	Reference
CAA:	
- circulating anodic antigen	32
- also known as SCA,GASP and GASCAP	33-35
- negatively charged	36
- the carbohydrate chains consist of multiple disaccharide units containing *N*-acetyl-galactosamine and glucuronic acid	37
CCA:	
- circulating cathodic antigen	32
- also known as 'antigen M'	38,39
- neutral or slightly positively charged	32
- the carbohydrate chains consist of multiple trisaccharide units (lewis x) containing fucose, galactose and *N*-acetyl-galactosamine	40
- contains some epitopes crossreactive with *Schistosoma* eggs	41
Both antigens are:	
- genus specific	39,42
- TCA soluble	32,39,42
- heat resistant	32,36,39
- sensitive to periodate	32,39,42
- produced by the gut epithelium of the adult worm	43-46
- also present in the primordial gut cells of cercariae and young worms	45-47
- demonstrated in the host's:	
serum and urine	32,36,38,48
milk (CCA only)	49
kidney, liver and spleen	44,50-52
- extremely stable, as illustrated by the detection of CAA in mummy tissue	53,54

[1] Part of this table has been adapted from the thesis of Van Dam.[55]

Additionally, specific antibody profiles are also of interest for research purposes, to study specific immune responses. Immuno-epidemiological studies revealed an association between the levels of specific IgE and resistance against reinfection, following chemotherapy. On the other hand susceptibility seems to be associated with an IgG4 response.[56,57]

Living parasites excrete and secrete a number of different antigens into the circulation of the host. These antigens can be classified, according to the life-stage of the parasite, into cercarial antigens,[58,59] adult worm associated antigens (e.g. tegument,[60,61] or gut-associated[62]), and egg antigens (mainly released as hatching fluid[63-65]).

The detection of these antigens in the circulation of the host potentially offers a large number of diagnostic, epidemiological and research applications: *a.o.* the quantitation of worm burden, assessment of chemotherapeutic efficacy and the impact of control measurements. Furthermore, circulating antigen detection could provide, in addition to egg counts, complementary information on the dynamics of parasite populations. It could, for example, be used for the evaluation of future vaccine candidates, especially if these vaccines also have an impact on the egg production of the parasites, which would make parasitological diagnosis unreliable.

Several studies have shown that the major circulating antigens belong to the group of the adult worm gut-associated circulating antigens. These antigens are released in the circulation of the host by regular regurgitating of the undigested contents of the parasites gut. Most research has concentrated on two glycoconjugates: circulating anodic antigen (CAA) and circulating cathodic antigen (CCA).[62,66] Both antigens are named after their electrophoretic mobility at neutral pH. The characteristics of CAA and CCA have recently been extensively reviewed by Van Dam.[55] Table 1 shows a summary of their most important properties.

CAA was first described by Berggren and Weller, and has been further characterised by Gold *et al.*, Nash *et al.*, and Deelder *et al.*.[32,34,36,42,44,48] It was recently found by Bergwerff *et al.* that the major carbohydrate chains of this proteoglycan have a novel polysaccharide structure.[37]

CCA, or 'antigen M', was independently found by Carlier *et al.* and Deelder *et al.*, and further characterized by these two groups.[32,38,39,44,67,68] Carbohydrate analysis has recently shown that this glycoprotein consists of a population of polysaccharides, containing repeating units of the Lewis-X trisaccharide.[40]

Table 2. Review of data on the demonstration of CAA and CCA in animals, experimentally infected with *Schistosoma* species which are infectious to humans.[1)]

Animal/ *Schistosoma* sp.	A cercs	antigen	B from week	C worm load	D treatment	Ref.
Mouse/						
S. mansoni	25-200	CCA	n.d.	yes	1 week	69
S. mansoni	25-250	CCA	5	yes	n.d.	70
S. mansoni	60-120	CAA	1	yes	1 week	71[3)]
S. mansoni	10-100	CAA	3	yes	n.d.	72
S. mansoni	100	CAA	5	no	n.d.	73
S. mansoni	100	CCA	n.d.	no	n.d.	73
S. mansoni	600	CAA	4	n.d.	n.d.	[4)]
S. mansoni	1000	CCA	2½	no	n.d.	74
S. mansoni	1000	CAA	2½	no	n.d.	74
S. japonicum	25	CCA	n.d.	yes	n.d.	73
S. japonicum	25	CAA	2	yes[2)]	n.d.	73
S. japonicum	5-28	CAA	4	yes	n.d.	72
S. haematobium	50-500	CAA	5	yes	n.d.	72
S. intercalatum	25-75	CAA	5	yes	n.d.	72
Baboon/						
S. mansoni	1500	CAA	5	no	n.d.	[4)]
S. mansoni	1500	CCA	5	no	n.d.	[4)]
S. haematobium	1500	CAA	6	n.d.	n.d.	[4)]

Abbreviations: sp. = species; A = number of cercariae; B = detectable, in weeks after infection; C = correlation with worm load; D = start decrease, in weeks after treatment with chemotherapy; Ref. = reference number; n.d. = not done.

[1)] Part of this table has been reproduced from Van Dam *et al.*.[74] To obtain comparable sensitivity and specificity, this table only summarizes data on the detection of CAA and CCA by monoclonal antibody based assays.

[2)] Only correlating with the number of female worms.

[3] This study contrasts with others with respect to the kinetic of CAA detectability; it seems unlikely that CAA is already detectable 1 week after infection with 120 cercariae and reaches maximum level already 2 weeks after infection, when the worms are still very small and the gut not yet fully developed.
[4] Van Lieshout *et al.*, unpublished data.

The primary function of both antigens is not exactly known. Based on their highly glycosylated structure, they probably play a role in the protection of the schistosome gut.[43] Recently, it has been argued that they may also protect the worm against cellular immune effector mechanisms of the host, as CAA interacts with the first complement component C1q,[75] and CCA presumably evokes an autoimmune response due to similarity with a major granulocyte surface antigen.[76]

Several techniques for the demonstration of these circulating antigens have been reviewed by De Jonge.[77] Initially, these assays were based on the use of polyvalent antisera. The development of monoclonal antibody (MAb) based sandwich ELISA's has made it possible to detect CAA and CCA in a sensitive and highly specific manner.[78,79] These MAb's have also been applied in some other techniques, e.g. for a more field applicable demonstration of these antigens.[80-84]

Table 2 summarizes the available data on the detection of CAA and CCA in experimental animal models. Generally, these antigens can be demonstrated in the circulation approximately 3 to 5 weeks after infection, depending on the intensity of infection. A significant correlation between antigen level and the number of adult worms after perfusion, was found in all studies infecting with different doses of cercariae. The relation between serum CAA level and worm burden was found to be linear and not affected by suppression of the cellular immune system.[72] Following successful chemotherapy, antigen levels decrease rapidly.

Initially, human studies were mainly focused on the detection of CAA in serum for the diagnosis of schistosomiasis mansoni. However, in later studies both 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. Table 3 summarizes the current data on the detection of CAA and CCA in humans, including the research described in chapter 2 of this thesis. In most studies, antigen levels were found to correlate significantly with the egg excretion and to decrease rapidly following successful treatment.

Table 3. Review of studies on the detection of CAA and CCA in human serum and urine.[1) 2)]

		CAA		CCA	
***Schistosoma* species:**		serum	urine	serum	urine
S. mansoni	detected	[3)]78,81, 85-90	88,89,91	79,85,86, 89,92-94	79,85,89, 91,92
	correlation[4)]	78,81 85-90	89,91	[5)]85,86, 89,92-94	85,89, 91,92
S. haematobium	detected	95	91,95	95	95
	correlation	95	95		95
S. intercalatum	detected	96,97	97	97	97
	correlation	96,97			97
S. japonicum	detected	98,99		99	
	correlation	98			
Specific studies:					
Prevalence and intensity in an endemic area		100-102	100	100	100,102
Follow up after chemotherapy		88,90,95, 98,101, 103-106	88,95, 104	93-95, 104,107	95,104, 107
Acute infections/Individuals from non-endemic area's		106,108, 109	109	109	109
Measurement of worm burden		110,111		110,111	

[1)] To obtain comparable sensitivity and specificity, this table only summarizes data on the detection of CAA and CCA by monoclonal antibody based assays. For a review of antigen detection in humans including assays based on polyvalent anti-sera, see De Jonge.[77]

[2)] This thesis includes the references: 88,89,100,104,107,109-111.

[3)] Reference number.

[4)] Studies which found a significant correlation between antigen levels and egg counts.

[5)] Reference 86 A correlation between antigen level and epg was only found for cases with intestinal schistosomiasis, not for cases with the hepatosplenic form of the disease.

The main objectives of the research described in this thesis concern the following three aspects of the determination of CAA and CCA in human serum and urine samples: (i) to investigate the application of these antigen assays for the diagnosis of an active *Schistosoma* infection, (ii) to further evaluate the use of these antigen assays for the assessment of cure following chemotherapy, and (iii) to use these antigen assays for more epidemiologically oriented studies, i.e. for the assessment of worm burdens to study infection patterns in endemic areas.

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Chapter 2.1

Improved diagnostic performance of the circulating antigen assay in human schistosomiasis by parallel testing for circulating anodic and cathodic antigens in serum and urine.

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Erratum:

page 30 - *Statistical methods* -
The fourth sentence should be changed into: *Properties of circulating antigen assays were evaluated by chi-square analysis including Yates' correction and by the McNemar test.*

page 31 - *Results* -
The last sentence of the second paragraph should be changed into: *The difference in sensitivity between the four circulating antigen assays was not significant (McNemar, $P > 0.29$; $n = 68$).*

IMPROVED DIAGNOSTIC PERFORMANCE OF THE CIRCULATING ANTIGEN ASSAY IN HUMAN SCHISTOSOMIASIS BY PARALLEL TESTING FOR CIRCULATING ANODIC AND CATHODIC ANTIGENS IN SERUM AND URINE

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Abstract. Serum and urine levels of two *Schistosoma* circulating antigens, the circulating anodic antigen (CAA) and the circulating cathodic antigen (CCA), were determined by monoclonal antibody–based enzyme-linked immunosorbent assays in 56 Egyptian patients infected with *S. mansoni* and in 12 patients infected with both *S. mansoni* and *S. haematobium*. Both CAA and CCA could be specifically demonstrated in 82% and 88% of the serum samples and in 88% and 87% of the urine samples, respectively. While complete specificity was maintained, sensitivity was increased to a range of 91–98% by parallel use of the two circulating antigen assays, i.e., an individual with a positive titer for at least one of the assays was considered to be infected. A combination of CAA and CCA determinations in urine samples only resulted in a sensitivity of 94%. However, the highest sensitivity was achieved when the serum-CCA assay was combined with the urine-CCA assay (98%) or with the urine-CAA assay (97%). Sensitivity could not be increased further by combining more than two tests. A significant correlation was demonstrated between the level of circulating antigen and the number of parasite eggs in feces in each of the four assays. In addition, the levels of CAA and CCA in serum and urine were significantly correlated with each other. Our results indicate that diagnosis of schistosome infections by detection of circulating antigens can be significantly improved by parallel testing for multiple antigens.

The detection of circulating antigens has been shown to be a promising approach for the diagnosis of human schistosome infections.[1–3] Most studies of this technique have concentrated on the quantitation of circulating anodic antigen (CAA), an adult worm gut–associated proteoglycan with a strong negative charge at neutral pH.[4–7] This antigen can be demonstrated in the serum and urine of patients infected with *Schistosoma mansoni, S. haematobium, S. japonicum,* and *S. intercalatum,* and the level of CAA shows a significant correlation with the intensity of infection. False-positive results have not been obtained with either body fluid.[8–11] The urine assay has the advantage of involving a noninvasive method of sampling, but it requires a time-consuming concentration technique because of the low level of CAA in urine.

Another major circulating adult worm antigen that has been described is circulating cathodic antigen (CCA), a gut-associated glycoprotein that is positively charged at neutral pH.[6, 12, 13] This antigen has also been demonstrated in the serum and urine of schistosomiasis patients,[3, 14, 15] but the number of studies on human samples has been limited because a sensitive enzyme-linked immunosorbent assay (ELISA) for the quantification of CCA was not developed until recently.[16]

No study has been performed in which the two circulating antigen assays were compared for both serum and urine in the same patient group, which would allow an evaluation of the diagnostic performance of the individual assays. In addition, an improvement in sensitivity is needed, especially for the diagnosis of schistosomiasis patients with a light infection and for monitoring after chemotherapy. One way to achieve increased sensitivity is by combining test results of highly specific assays. In this method of parallel testing, a person is diagnosed as being infected when at least one of the applied assays shows a positive result.[17] This approach seems to be applicable to the schistosome circulating antigen assays because of their high specificity.

The purpose of the present study was to compare the two circulating antigen assays for both serum and urine and to analyze which circulating

antigen assay or combination of assays is most sensitive for diagnosing a *Schistosoma* infection. We examined the levels of both CAA and CCA in serum and urine samples of a group of Egyptian schistosomiasis patients.

PATIENTS AND METHODS

Study population

Urine and serum samples of 68 Egyptian schistosomiasis patients were tested for levels of CAA and CCA. All patients were males and ranged in age from 10 to 49 years (median 17 years). Fecal and urine egg excretion were determined by calculating the mean of three consecutive duplicate 25-mg Kato Katz slides and of two consecutive 10-ml Bell examinations, respectively.[18, 19] All patients had live *S. mansoni* eggs in their stool. Twelve also showed live *S. haematobium* eggs in their urine.

Levels of CAA and CCA were also determined in 28 serum and 56 urine samples collected from 11 healthy endemic controls, who showed no clinical symptoms of schistosomiasis. These individuals were parasitologically negative when repeatedly tested for *Schistosoma* eggs in feces and urine, and were also negative for IgM-specific antibodies when tested by an indirect immunofluorescence assay using paraffin sections of Rossman's fixed *S. mansoni* worms.[20]

Informed consent was obtained from all individuals who participated in this study.

Collection and treatment of samples

Serum and urine samples were collected at the same time and were stored frozen at −20°C until use. All samples were pretreated with trichloroacetic acid (TCA) to remove interfering proteins and to dissociate immune complexes.[21] Samples were tested at an initial dilution of 1/4, except for urine samples tested for CAA, which were lyophilized and reconstituted in buffer to give a five-fold increase in concentration of the original samples.[10] Each sample was assayed once in a single-blinded fashion.

Determination of circulating antigens

Circulating anodic antigen was assessed by the ELISA described by Deelder and others using an anti-CAA IgG_1 mouse monoclonal antibody as coating (antigen-capture) antibody and as the alkaline phosphatase–conjugated secondary (antigen-detecting) antibody.[8] Urine and serum samples were tested in two-fold dilution series. The reciprocal value of the last sample dilution showing color above the background level (mean absorbance + 2 SD of buffer controls) was taken as the titer. Consistent with previous findings, serum samples were considered to be negative for CAA at a titer < 4 and positive at a titer ≥ 4. For urine samples, these titers were < 0.2 and ≥ 0.2, respectively. The lower detection limit of this ELISA was 0.8–1.6 ng/ml of the TCA-soluble fraction of adult worm antigen.

Circulating cathodic antigen was quantitated by the ELISA described by De Jonge and others with minor modifications.[16] Test wells were blocked with a solution of 0.1% (w/v) bovine serum albumin (BSA) and the assay buffer contained 0.5% (w/v) BSA. In this assay, an anti-CCA IgG_3 mouse monoclonal antibody was used for coating, while a biotinylated IgM mouse monoclonal antibody was used as the secondary antibody. Streptavidin-alkaline phosphatase was used as enzyme label. Titers were determined as for CAA, except that the background level was calculated by the mean absorbance plus 2 SD of a 1/4 dilution of a TCA-treated negative serum or urine pool. The individual samples of these pools had been previously shown to be negative for CAA.[9, 22] The lower detection limit of this ELISA was 3.9–7.8 ng/ml of the TCA-soluble fraction of adult worm antigen.

Statistical methods

Since circulating antigen titers and schistosome egg output did not show a continuous or normal distribution, non-parametric statistical methods were used to describe and evaluate the results. Data were characterized by their ranges and medians. Kendall's W and Spearman's rank test were used for testing concordance. Properties of circulating antigen assays were evaluated by chi-square analysis including Yates' correction. The tests were run on an IBM PC/XT compatible computer using the SPSS/PC$^+$ statistical package (SPSS Inc., Chicago, IL).

RESULTS

The fecal egg output of the 56 *S. mansoni* patients ranged from 10 to 976 eggs per gram of

feces (epg) (median 167 epg). In the 12 patients with mixed infections, the fecal egg output ranged from 13 to 1,213 epg (median 209 epg). Urine egg counts ranged from 0 to 340 eggs per 10 ml of urine (ep 10 ml) (median 9 ep 10 ml).

All serum and urine samples collected from the 11 healthy individuals showed negative titers for CAA. In the serum-CCA assay, 27 of the 28 collected samples had titers ≤ 4 and one had a titer of four. In the urine-CCA assay, 45 of the 56 samples had titers ≤ 4, five had titers of four, and six had titers of eight. Therefore, serum and urine samples with titers ≥ 8 and ≥ 16, respectively, were considered to be positive for CCA.

The results of screening serum and urine samples of 68 schistosomiasis patients for the presence of CAA and CCA are summarized in Table 1. The sensitivity of the CAA and CCA assays was 82% and 88% for serum and 88% and 87% for urine, respectively. The sensitivity of the serum-CAA assay was significantly lower than that of the three other assays ($\chi^2 > 7.5$, $P < 0.01$; n = 68).

Table 1 also shows the data of patients infected with *S. mansoni* only, both in individuals with a light infection (passing less than 100 epg) and in those with a moderate to heavy infection (passing more than 100 epg). In the light infection group, the sensitivity of the individual assays ranged from 75% to 85%, while in the moderate to heavy infection group, the sensitivity ranged from 86% to 94%. The difference in sensitivity between the two infection groups was not significant ($\chi^2 < 2.8$, $P > 0.05$; n = 56).

The results of parallel testing with combinations of two tests by which patients had been considered positive based on a positive titer for either of the antigen assays are shown in Table 1. Sensitivity was increased for all combinations, and ranged from 91% to 98%. When urine CAA and CCA determinations were combined, 94% of the patients were positive. The highest sensitivity was achieved when the serum-CCA assay was combined with the urine-CCA assay (98%) or with the urine-CAA assay (97%). Sensitivity could not be improved by combining more than two tests, since one patient, with an egg count of 10 epg, was negative by all four assays. Forty seven (69%) patients were positive by all four assays. In the group with light infections, combined assaying increased the sensitivity to a range of 85–95% and nine (44%) patients were positive by all four assays. In the group with moderate

TABLE 1

No. (%) of schistosomiasis patients positive by enzyme-linked immunosorbent assays for the detection of circulating anodic antigen (CAA) or circulating cathodic antigen (CCA) in serum or urine or in a combination of two of these assays

Group	n	Serum CAA	Urine CAA	Serum CCA	Urine CCA	Serum CAA plus urine CAA	Serum CAA plus urine CCA	Serum CAA plus serum CCA	Urine CAA plus urine CCA	Urine CAA plus serum CCA	Serum CCA plus urine CCA
All patients*	68	56 (82)	60 (88)	60 (88)	59 (87)	62 (91)	63 (93)	63 (93)	64 (94)	66 (97)	67 (98)
Schistosoma mansoni infections	56	46 (82)	49 (88)	51 (91)	47 (84)	51 (91)	51 (91)	53 (95)	52 (93)	55 (98)	55 (98)
≤ 100 epg†	20	15 (75)	15 (75)	17 (85)	15 (75)	17 (85)	17 (85)	19 (95)	17 (85)	19 (95)	19 (95)
> 100 epg	36	31 (86)	34 (94)	34 (94)	32 (89)	34 (94)	34 (94)	34 (94)	35 (97)	36 (100)	36 (100)

* Fifty-six *S. mansoni*-infected patients and 12 patients infected with both *S. mansoni* and *S. haematobium*.
† epg = eggs per gram of feces.

TABLE 2

Coefficient of correlation between circulating anodic antigen (CAA) and circulating cathodic antigen (CCA) levels in serum and urine samples of 68 schistosomiasis patients and between egg output and antigen level of 56 patients infected with Schistosoma mansoni*

	Serum CAA	Urine CAA	Serum CCA	Urine CCA	Egg output
Serum CAA	1.00	0.83	0.52	0.65	0.57
Urine CAA	–	1.00	0.55	0.66	0.61
Serum CCA	–	–	1.00	0.46	0.57
Urine CCA	–	–	–	1.00	0.48

* $P < 0.001$ for all coefficients.

to heavy infections, sensitivity increased to a range of 94–100%, while 29 (80%) of the 36 patients showed positive titers by all four assays.

A significant correlation was found between the number of parasite eggs in the feces of patients infected with *S. mansoni* and the level of circulating antigen for each assay (Spearman's $\rho > 0.47$, $P < 0.001$; n = 56) (Table 2). The highest correlation coefficient was found with CAA in urine ($\rho = 0.61$), and the lowest was found with CCA in urine ($\rho = 0.48$). The coefficient of concordance (Kendall's W) for the four assays was 0.71 (n = 68; $P < 0.001$). Table 2 also shows the Spearman's rank coefficients for each combination of two assays; all were significant ($P < 0.001$). The highest correlation coefficient was found between the CAA titers in serum and urine ($\rho = 0.83$), and the lowest was found between CCA in serum and urine ($\rho = 0.46$).

Figure 1 depicts the relationships between serum and urine antigen levels of CAA and CCA, respectively, for all patients tested. While low to moderate CAA titers were seen in the eight patients positive by one of the two CAA assays, moderate to high CCA titers were found in the 15 patients positive by one of the two CCA assays.

DISCUSSION

The demonstration in serum or urine of schistosomal CAA has been shown to have a strong diagnostic potential.[8-11, 14, 15, 22] For patients in endemic areas, the sensitivity of the CAA assay was found to range from 100% in patients passing more than 400 epg to 60% in patients passing less than 100 epg.[9] Thus, for diagnosing light infections and also for monitoring the efficacy of chemotherapy in individual patients, an improvement of sensitivity is still needed.

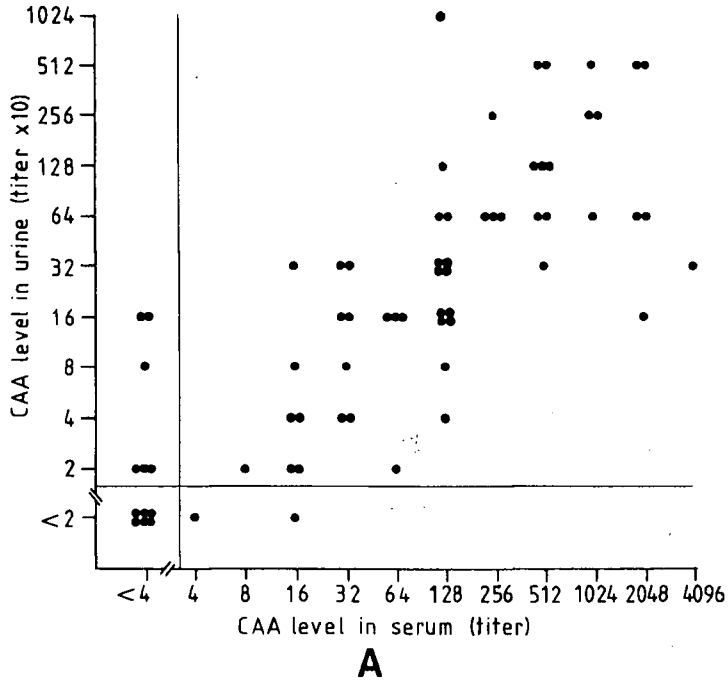
For CCA, the number of studies on human schistosomiasis patients has been limited.[3, 14-16] In a recent study on serum samples of Brazilian patients with *S. mansoni* infections, the sensitivity of the CCA assay (83%) was found to be significantly higher than that of the CAA assay (66%) ($\chi^2 = 13.6$, $P < 0.01$; n = 58).[15] However, no studies were performed to compare the two circulating antigen assays for both serum and urine.

In this study, we evaluated the CAA and CCA assay for two body fluids and combined the results to evaluate diagnostic performance. Before analyzing schistosomiasis patients, serum and urine samples of 11 healthy individuals were tested for CAA and CCA to verify the specificity of both antigen assays. None of these individuals showed *Schistosoma* eggs in feces or urine, or specific IgM in serum. Consistent with previous results, no CAA was found in their serum or urine.[8-11, 22] However, low titers were found in the CCA assay. For this reason, serum and urine samples in this study were considered positive for CCA if samples had titers ≥ 8 and ≥ 16, respectively. Previously, this positive titer value was ≥ 4 for both body fluids.[16]

Both CAA and CCA were found in serum and urine samples of 68 Egyptian schistosomiasis patients. We found the sensitivity of the serum-CAA assay (82%) to be significantly lower than that of the serum-CCA assay (88%), which is consistent with a previous study.[15] The sensitivity of the serum-CAA assay was also significantly lower than the sensitivity of the urine CAA (88%) and the urine CCA (87%) assay.

By using two circulating antigen assays in parallel, the sensitivity was increased to a range of 91–98%, but no additional increase in sensitivity was observed by combining more than two assays. The highest sensitivity was achieved by combining the serum-CCA assay with the urine-CCA assay (98%) or with the urine-CAA assay (97%). However, the use of only urine samples would be highly desirable for mass population–screening programs. By combining the CAA and CCA determinations for urine samples, a sensitivity of 94% was achieved.

In this study, only male subjects were tested, and this rules out the possible influence of vaginal excretion, feces, or menstrual blood on the sensitivity or specificity of the urine assays. How-



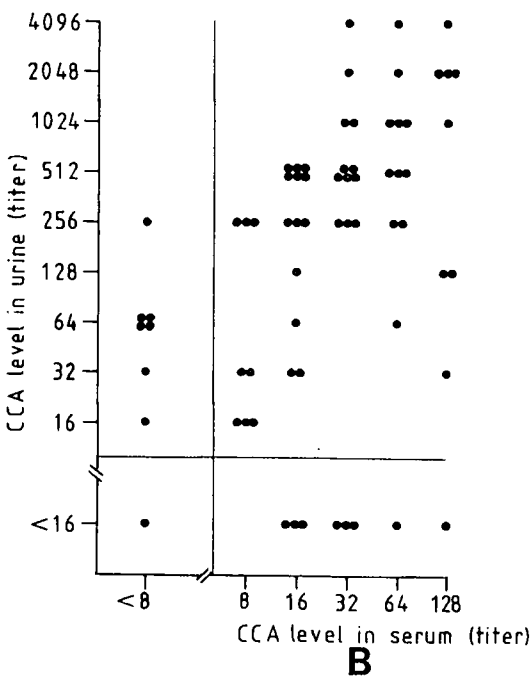


FIGURE 1. Correlation between serum and urinary levels of **A**, circulating anodic antigen (CAA) or **B**, circulating cathodic antigen (CCA) in 68 patients infected with *Schistosoma mansoni* or with a mixed infection of *S. mansoni* and *S. haematobium*. The lines indicate the negative/positive cut-off values.

ever, no sex-related differences were observed in a previous study in which urine samples collected from both male and female individuals were tested for their CAA level.[10] The serum-CCA assay was found to be the most sensitive test in patients with light infections (< 100 epg). It also gave the best diagnostic performance when combined with any of the other assays.

For each assay, a significant correlation was demonstrated between the level of circulating antigen and the egg count, which is consistent with previous findings.[9-11,22] The level of CAA and CCA in serum and urine were also significantly correlated with each other. As shown in Figure 1, the relatively low correlation coefficient between serum CCA and urine CCA levels resulted from the fact that several patients showed a moderate to high CCA level in serum and no CCA in urine, or vice versa; this was less frequent with CAA. This difference between CAA and CCA levels could not be explained by experimental variation. We previously demonstrated for the CAA and CCA assays an intra-assay variation of 6% and 8% and an interassay variation of 15% and 10%, respectively.[8,16] A possible explanation could be a difference in the clearance mechanism of the two antigens. The clearance rate is determined by the formation and size of immune complexes. In turn, both of these are influenced by the ratio between antigen and antibody, which could vary individually with the state of infection.[23,24] Recently, it has been shown that schistosome-specific IgM antibodies are directed mainly against CCA and not CAA.[25] To gain a better understanding of these mechanisms, further studies should be performed on the relationship between the humoral immune response, the formation of immune complexes, and the clearance rate of these two antigens.

In conclusion, this study demonstrates that the diagnosis of human schistosomiasis by quantification of circulating antigens can be improved by parallel testing for more than one antigen and in more than one body fluid. Whether parallel testing also benefits monitoring of failure of treatment will be determined.

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Chapter 2.2

Evaluation of an ELISA for combined measurement of CAA and CCA in schistosomiasis mansoni.

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Evaluation of an ELISA for combined measurement of CAA and CCA in schistosomiasis mansoni

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An enzyme-linked immunosorbent assay was developed for combined measurement of schistosome circulating anodic antigen (CAA) and circulating cathodic antigen (CCA). Monoclonal antibodies against CAA and CCA were used as coating and as fluorescein-labeled detecting antibodies in a FITC–anti-FITC system. The lower detection limit of the assay was 1.1 ng antigen (AWA-TCA)/ml. Serum samples of *Schistosoma mansoni* infected individuals from Zaire ($n=60$) and Burundi ($n=60$) were tested in this assay and in single-antigen ELISAs. Sensitivities of assaying for CAA, CCA, combined CAA+CCA, and of parallel testing for CAA and for CCA were calculated from titres and antigen concentrations. With serum samples from the heavily infected individuals (Zaire), all assays had a sensitivity of 97% or higher. In contrast, with serum samples from individuals from Burundi (low to moderate infections) it was shown that combined testing resulted in a slightly lower sensitivity than testing for individual antigens. By parallel testing for CAA and CCA, the sensitivity could be increased considerably (to 95%), however.

Key words: Human schistosomiasis; Combined ELISA; Circulating anodic antigen; Circulating cathodic antigen; FITC–anti-FITC system

Introduction

For the quantitative diagnosis of schistosomiasis, much research has been focused these last few years on the development of assays for detection of circulating schistosome adult worm antigens in serum of patients. Two of the best studied antigens are the circulating anodic antigen, CAA (Berggren and Weller, 1967; Nash et al., 1974; Deelder et al., 1976; Feldmeier et al., 1986; Deelder et al., 1989; De Jonge et al., 1988), and the circulating cathodic antigen, CCA (Carlier et al., 1975; Deelder et al., 1976; Feldmeier et al., 1986; De Jonge et al., 1990). Although it has been shown in several studies that the sensitivity of sandwich ELISAs for detection of CAA is similar to that of stool examination by a three times repeated duplicate Kato–Katz smear of 25 mg, especially in light infections, false-negative results do occur (De Jonge et al., 1988). In principle, sensitivity might be increased by applying assays with an 'increased' lower detection level, or, as recently shown by Van Lieshout et al. (1992), by parallel testing for two antigens — in that case CAA and CCA — and combining the data. As it would be advantageous to realize this same

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increase in sensitivity in one assay, here we report the application of a combined ELISA for simultaneous detection of both CAA and CCA in one sample. Because in the originally described assays, different conjugation procedures had to be applied for the respective monoclonals used, we have applied the FITC–anti-FITC system (Samuel et al., 1988; Harmer and Samuel, 1989) to enable a combined assay.

Materials and methods

Study population

Serum samples were studied from two groups of individuals. Group 1 consisted of 60 infected individuals from Maniema, Eastern Zaire. Group 2 consisted of 60 infected individuals from an endemic area in Burundi. These groups of patients have been described in detail by Polderman et al. (1985a; 1985b) and Gryseels (1984), respectively. From the total of 120 people 116 excreted *Schistosoma mansoni* eggs, while the remaining 4 had in a previous study (De Jonge et al., 1988) been determined as active infections by circulating antigen detection.

Serum samples from 72 Dutch individuals, with no history of schistosomiasis, were taken as negative controls.

Parasitological examination

Faecal egg excretion in all patients was determined by three consecutive duplicate 25 mg (Zaire) or 28 mg (Burundi) Kato–Katz examinations (Katz et al., 1972). Blood samples were taken at the third egg count.

Pretreatment of sera with TCA

Before testing, sera were treated with trichloroacetic acid (TCA), to remove proteins and to dissociate immune complexes as previously described (De Jonge et al., 1987).

Use of monoclonal antibodies in the ELISA

For the CAA determinations, the assay was based on the ELISA described by Deelder et al. (1989), now using the mouse monoclonal antibody (mAb) 120–1B10-A (anti-CAA, IgG_1) as coating and as fluorescein-labeled detecting antibody. For the CCA-ELISA, based on the assay described by De Jonge et al. (1990), mouse mAb 54–5C10-A (anti-CCA, IgG_3) was used as coating antibody and mouse mAb 8.3C10 (anti-CCA, monomeric IgM) as fluorescein-labeled detecting antibody. To detect both CAA and CCA simultaneously the coating solution consisted of both 120–1B10-A mAb and 54–5C10-A mAb, while a mixture of 120–1B10-A/fluorescein and 8.3C10/fluorescein was used in the detecting antibody step.

A description of the production of mouse mAb 256–4A1-A (anti-FITC, IgG_1), in this study conjugated to alkaline phosphatase, is given by Van Dam et al. (1991).

Conjugation of monoclonal antibodies

mAb 120–1B10-A (anti CAA) and mAb 8.3C10 (anti CCA) were labeled with activated fluorescein, 5(6)carboxyfluorescein-*N*-hydroxysuccinimide ester (FLUOS, Boehringer, F.R.G.), following the procedure given by the manufacturer for labeling mAb. In short, 0.064 mg FLUOS (concentration 2 mg/ml) was added to 2 mg mAb dissolved in 2 ml PBS during stirring and incubated for two hours at room temperature during gentle stirring, protected from light. Remaining non-reacted FLUOS was separated by gel filtration on a prepacked PD10 Sephadex G-25 column (Pharmacia). The F/P ratio was 4.0 for 120–1B10-A/fluorescein and 5.9 for 8.3C10/fluorescein. The fluorescein-labeled mAbs were stored at −20°C in small aliquots.

mAb 256–4A1-A (anti-FITC) was conjugated to alkaline phosphatase (Boehringer) following the glutaraldehyde method described by Engvall and Perlmann (1971). Anti-FITC/AP was stored at −20°C in 50% (v/v) glycerine. The fluorescein–anti-fluorescein system is further designated by its commonly used name: FITC–anti-FITC system.

Determination of CAA, CCA and the combination of CAA and CCA

After optimization of the test conditions, the following ELISA procedure was adopted: polystyrene flat-bottom microtitration immunoassay plates (Maxisorp, NUNC, Denmark) were coated with mAb(s) by adding 100 µl of 5 µg mAb per millilitre in 0.035 M phosphate-buffered saline (PBS), pH 7.8. To detect both CAA and CCA simultaneously the optimal coating solution consisted of 1.25 µg anti-CAA mAb and 3.75 µg anti-CCA mAb per millilitre. Plates were incubated for three hours at 37°C and washed with 0.002 M PBS. The wells were postcoated for 1 h at 37°C with 120 µl 330 µg/ml BSA (Boehringer, fraction V) in PBS pH 7.8 and washed. Sera were tested in a two-fold dilution series, starting with a four times diluted sample. Dilutions were made in assay buffer: 0.035 M PBS pH 7.8+0.02% (v/v) Tween 20 (Sigma, St. Louis, MO) + 0.5% (w/v) PEG 1000 (Merck, Schuchardt)+0.1% (w/v) BSA (Boseral, Organon Teknika, The Netherlands). Each well received 80 µl and was incubated for 1 h at 37°C and washed. Eighty µl of 0.50 µg/ml fluorescein-labeled mAb, or a 1:1 mixture of two mAbs, was added to each well and incubated for 1 h at 37°C and washed. mAb 256–4A1-A (anti-FITC) conjugated to alkaline phosphatase was diluted 160-fold in assay buffer (from the stock solution in glycerol) added, 80 µl/well, and incubated for 1 h at 37°C. After washing, 80 µl 3 mM 4-nitrophenyl phosphate (Boehringer) was added, diluted in 0.1 M diethanolamine containing 0.5 mM $MgCl_2$, pH 9.6. Substrate incubation was overnight at 4°C. Absorbance was read at 405 nm with an automated microplate reader, EL 311 (Biotek Instruments, U.S.A.).

Result interpretation

Results were expressed as titres as well as concentrations. For the titre the value of the last sample dilution showing a colouring above background level was taken. The concentration was calculated from the four-parametrical fitted curve of the data of a standard dilution series of the TCA-soluble fraction of adult worm antigen

(AWA-TCA). The software Kineticalc from Biotek Instruments, U.S.A., was used. For the background level, the mean absorbance plus three times the standard deviation of a number of buffer controls was taken.

Statistical methods

Data were characterized by their medians and ranges. To evaluate differences between sensitivities, 95% confidence intervals were calculated. Because neither egg output, nor circulating antigen concentrations are normally distributed, non-parametric statistical methods were used. Spearman's rank and Kendall's *W* test were used to calculate concordance between two and three variables, respectively. The statistical software SPSS/PC+ (SPSS Inc., Chicago, IL) was used on an IBM/XT compatible personal computer.

Results

Using the FITC–anti-FITC system, for the CAA ELISA it was found that the lower detection limit was 1.5 ng AWA-TCA per millilitre and for the CCA ELISA 1.8 ng AWA-TCA per millilitre (CAA and CCA each constitute approximately 3% of total AWA-TCA (Van Dam, et al., 1993). The combined ELISA, detecting both CAA and CCA in one sample, had a lower detection limit of 1.1 ng AWA-TCA per milliliter. Typical AWA-TCA dose response curves for these three assays are shown in Fig. 1.

For the CAA ELISA, samples with a reciprocal titre ≥ 4 and a concentration ≥ 6

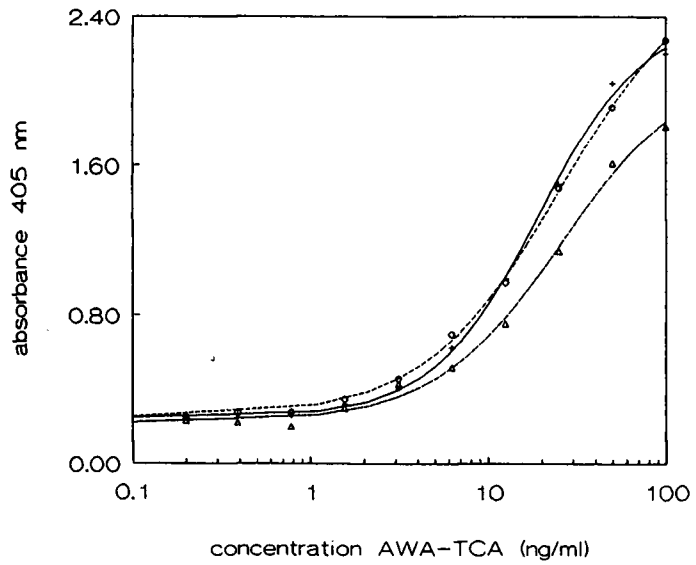

Fig. 1. Dose response curves of three ELISAs for detection of circulating anodic antigen (CAA; +), circulating cathodic antigen (CCA; △) and combined CAA+CCA (○), as determined with a dilution series of the trichloroacetic acid-soluble fraction of adult worm antigen (AWA-TCA). Curves were fitted by a four-parameter fit.

ng AWA-TCA/ml were considered to be positive. All 72 negative control sera showed a reciprocal titre smaller than 4, thus resulting in an assay-specificity of 100%. In both the CCA ELISA and the combined ELISA, all but 4% of the negative control sera showed reciprocal titres ranging from 2 to 16. Therefore reciprocal titres ≥ 32 were considered positive, resulting in an assay-specificity of 96%. In terms of concentration the cut-off values for the CCA assay and the combined assay were 58 and 32 ng AWA-TCA per milliliter, respectively.

The distribution of egg output and of circulating schistosome antigen levels in serum of individuals from both tested groups are given in Table 1. Differences in antigen levels were a reflection of differences in egg output for the two study groups. The range between minimum and maximum was more pronounced for CAA levels than for CCA levels, while the range of the combined antigen levels was in between these two ranges.

Analysis of the test results (Table 2) showed a clear difference in assay sensitivity for the two study groups. Titres and concentrations gave, only in the group with lower egg-output (Burundi), slightly different (but not significant) results of sensitivity. The combined ELISA, detecting both CAA and CCA in one sample, failed to show a higher sensitivity than the individual CAA or CCA assay. By parallel use of the two tests, the sensitivity was increased considerably.

For each assay, a significant correlation was found between egg-output and concentration (Spearman's $\rho > 0.50$, $P < 0.001$; $n = 60$) (Table 3). The highest correlation coefficient was found with the combined assay detecting CAA and CCA in one sample ($\rho = 0.74$). In both study groups the correlation for the CCA assay was lower than that for the CAA assay. Table 3 also shows the Spearman's rank coefficients for each combination of two assays. The correlation between the CAA assay and

TABLE 1

Distribution of egg output and circulating schistosome antigen levels in serum samples of two groups of *Schistosoma mansoni* infected individuals from Zaire ($n = 60$) and Burundi ($n = 60$)

	Zaire				Burundi			
	min	med	90-p	max	min	med	90-p	max
Epg	0	390	1134	9580	0	59	236	928
Reciprocal titres								
CAA	2	512	4096	8192	2	64	512	4096
CCA	64	256	1945	4096	4	64	256	512
[CAA,CCA]	32	1024	4096	8192	2	64	1024	2048
ng AWA-TCA/ml								
CAA	0	659	4214	7498	0	73	617	3865
CCA	67	348	1489	2528	7	145	361	612
[CAA,CCA]	35	539	3725	7502	0	81	446	2215

Antigen levels determined by ELISA and expressed as reciprocal titres and as concentrations (ng AWA-TCA/ml).

Abbreviations: AWA-TCA = the trichloroacetic acid-soluble fraction of adult worm antigen; min = minimum; med = median; 90-p = 90-percentile; max = maximum; Epg = eggs per gram faeces; CAA = circulating anodic antigen; CCA = circulating cathodic antigen; [CAA,CCA] = CAA and CCA detected in a combined assay.

TABLE 2

Sensitivities of assaying circulating schistosome antigens by ELISA in serum samples of *Schistosoma mansoni* infected individuals from Zaire ($n=60$) and Burundi ($n=60$)

	Zaire		Burundi	
	Se (%)	95% C.I.	Se (%)	95% C.I.
Titres				
CAA	97	(93–100)	77	(66–88)
CCA	100	(100)	85	(76–94)
[CAA,CCA]	100	(100)	75	(64–86)
CAA+CCA	100	(100)	95	(89–100)
ng AWA-TCA/ml				
CAA	97	(93–100)	77	(66–88)
CCA	100	(100)	80	(70–90)
[CAA,CCA]	100	(100)	68	(56–80)
CAA+CCA	100	(100)	93	(87–99)

Sensitivities calculated from titres and from antigen concentrations (ng AWA-TCA/ml).

Abbreviations: Se=sensitivity; 95% C.I.=95% confidence interval ($=p\pm 1.96\sqrt{p(1-p)/n}$, where p=positivity rate [i.e., sensitivity], and n=number of observations); AWA-TCA=the trichloroacetic acid-soluble fraction of adult worm antigen; CAA=circulating anodic antigen; CCA=circulating cathodic antigen; [CAA,CCA]=CAA and CCA detected in a combined assay; CAA+CCA=CAA and CCA determined by parallel assaying.

TABLE 3

Spearman's rank coefficient of correlation between concentrations of circulating schistosome antigens in serum and egg output of two groups of *Schistosoma mansoni* infected individuals from Zaire ($n=60$) and Burundi ($n=60$) ($P<0.001$ for all coefficients)

	Zaire			Burundi		
	CAA	CCA	[CAA,CCA]	CAA	CCA	[CAA,CCA]
CAA		0.67	0.98		0.54	0.91
CCA	—		0.74	—		0.75
[CAA,CCA]	—	—		—	—	
Epg	0.64	0.51	0.64	0.67	0.59	0.74

Abbreviations: CAA=circulating anodic antigen; CCA=circulating cathodic antigen; [CAA,CCA]=CAA and CCA detected in a combined assay; Epg=eggs per gram faeces.

the combined assay was the highest in both groups ($\rho>0.90$), while the correlation between the CAA assay and the CCA assay was the lowest. Kendall's coefficient of concordance for the three assays was 0.87 (Zaire) and 0.82 (Burundi) ($P<0.001$; $n=60$).

Discussion

For the quantitative diagnosis of schistosome infections, sensitive ELISAs for the detection of the adult worm antigens CAA and CCA have been developed. Although

such assays have a sensitivity which is comparable to 'classical' parasitological diagnosis, false-negative results occur. Van Lieshout et al. (1992) showed that the overall sensitivity of diagnosis by circulating antigen detection can further be improved by parallel testing for CAA and CCA. Thus, in a group of Egyptian patients, a sensitivity of 93% was achieved by combining the serum CAA assay with the serum CCA assay. The parallel testing for individual antigens has the obvious advantage of generating maximal information on the levels of individual antigens, allowing possible correlation with infection or morbidity parameters (De Jonge et al., 1991). For many applications, however, a positive/negative answer would be sufficient and it would therefore seem to be a logical step to evaluate the possibility of simultaneous detection of CAA and CCA in one assay. Each of the procedures which had been applied to labeling of the anti-CAA and anti-CCA antibodies, respectively direct alkaline phosphatase coupling and biotinylation, could not be applied to both mAbs without a significant loss of sensitivity in the single-antigen ELISAs (unpublished data), while in contrast both mAbs could be labeled succesfully with fluorescein (FITC). As FITC labeling of monoclonal antibodies is a simple technique, and the FITC–anti-FITC system is now increasingly used as an alternative to the biotin–avidin system (Wilchek and Bayer, 1988), we have evaluated whether this technique could be applied to allow detection of CAA and CCA in one assay. After optimization of the assay with purified CAA and CCA preparations, a lower detection level of 1.1 ng AWA-TCA in the combined assay was realized, which is in the same order of magnitude as the originally described assays for the individual antigens. Application of the assay to serum samples, disappointingly, did not result in the wished increase in sensitivity, resulting from a positive signal generated by the highest individual test. An evaluation of the results showed that this was mainly due to two reasons. Firstly, the samples containing CCA (and only little or no CAA) just above cut-off value were missed, because the detection level of CCA in serum was slightly decreased in the combined assay, compared to the CCA assay. This difference in detection was not found with purified CCA. This suggests that the antigen composition in purified CCA does not fully correspond to the composition of CCA in serum. Secondly, those samples with low CAA concentration (and without CCA) were missed, because in the combined assay concentrations below 30 ng/ml were considered negative, while the cut-off value for the CAA assay was 6 ng/ml. In contrast, it was shown that an increase in sensitivity could be achieved combining the results (i.e. parallel testing) of the individual assays, corroborating previous observations (Van Lieshout et al., 1992). From these results it can be concluded that the combination of two satisfactory target antigens in one assay does not necessarily guarantee success. Although individual CCA detection shows a high sensitivity, the relatively high cut-off level in comparison to the CAA assay, makes this antigen a less likely candidate for combined assaying. A combination of an assay for CAA with that for another antigen, preferably with a similar low cut-off level, might be a more promising alternative.

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Chapter 2.3

Assessment of cure in schistosomiasis patients after chemotherapy with praziquantel by quantitation of circulating anodic antigen (CAA) in urine.

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ASSESSMENT OF CURE IN SCHISTOSOMIASIS PATIENTS AFTER CHEMOTHERAPY WITH PRAZIQUANTEL BY QUANTITATION OF CIRCULATING ANODIC ANTIGEN (CAA) IN URINE

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Abstract. The kinetics of circulating anodic antigen (CAA) levels in urine were studied in Egyptian male patients infected with *Schistosoma mansoni* or with both *S. mansoni* and *S. haematobium*, before treatment, and at one, three and six weeks after chemotherapy. A quantitative enzyme-linked immunosorbent assay (ELISA) demonstrated CAA in 82% of the serum and 89% of the urine samples from these 28 patients. To evaluate the possibility of circadian variability in urine CAA levels, samples were examined in 15 patients at four intervals during a 24-hour period. No significant differences in CAA titers were observed. Seventeen patients were subsequently treated with praziquantel and followed for six weeks. CAA titers in serum and urine decreased significantly one week after therapy. Thereafter, the profile of CAA titer in urine continued to show a parallel but delayed decline compared to that in serum. While all serum CAA titers became negative three to six weeks after treatment, urine titers were negative in 47% at three weeks and 69% at six weeks. The remaining positive patients had low titers. A significant quantitative correlation in CAA titer was found between serum and urine before and after treatment. Seventeen Egyptian control subjects with no active schistosome infection were negative for CAA in both serum and urine. Our results confirm that the CAA urine assay could be used as a sensitive and non-invasive method to diagnose the disease, and indicate that the assay can be used to monitor efficacy of schistosome chemotherapy.

Quantitation of circulating antigens has been shown to be a promising alternative for the diagnosis of *Schistosoma* infections.[1-3] Several potentially diagnostic antigens have been previously described.[4, 5] One of the major *Schistosoma* antigens is CAA (circulating anodic antigen), also known as GASP (gut-associated schistosome proteoglycan). This antigen was originally described by Berggren and Weller,[6] and has been further characterized by several authors.[7-9] Circulating anodic antigen is an adult worm, gut-associated proteoglycan with a molecular weight of 70kDa and a strong negative charge at neutral pH.[8-10] It is heat stable and trichloroacetic acid (TCA) soluble. From immunoprecipitation studies the antigen was shown to be genus specific.[8, 11, 12] CAA is localized in the schistosome's gut epithelium.[13, 14]

ELISA has been proven to be a useful technique for the quantitative determination of CAA.[1, 2, 12] Serum antigen level correlated well with the host's worm burden as expressed by egg output,[15] and the effect of chemotherapy could be monitored in a period of weeks.[3]

Recently we reported the presence of the antigen in urine of patients infected with *S. mansoni, S. haematobium* or with mixed infection of both helminths.[16] Here we studied the effect of chemotherapy on CAA levels in the urine of patients infected with *S. mansoni* or with mixed infections of *S. mansoni* and *S. haematobium*.

MATERIALS AND METHODS

Patients

Patients and controls for this study were included after granting informed consent. Urine and serum samples of 28 Egyptian hospitalized patients were studied for CAA levels. They were all males and ranged in age from 10–49 years (median 18 years, 90th percentile 35 years). Twenty patients had live *S. mansoni* eggs in their stools and were treated with 3 doses of 20 mg/kg praziquantel (Biltricide) given orally at four- to six-hour intervals. Parasitological examination of stools was repeated three to six weeks after treatment. The other eight patients had mixed *S. mansoni* and *S. haematobium* infections.

Urine samples of 12 healthy Egyptians and

serum and urine samples of five hospitalized patients with no schistosomal infection were used as negative controls. Their ages ranged from 11–60 years (median 26, 90th percentile 34 years).

Parasitological examination

Fecal egg excretion in all patients was determined by counting the mean of three consecutive duplicate 25 mg Kato examinations, starting on the day of hospitalization.[17]

Bell's egg-count quantitation technique was used in two consecutive 10 ml urine samples.[18]

Collection of urine and serum samples

To study the circadian rhythm of urine CAA levels, samples were collected from 15 patients (of which seven had mixed infections) at four different times during the day, spread over a period of 5–12 days. Of the 13 other patients, 24-hour urines were collected only once. A blood sample was taken from all patients at the time of the first urine collection.

For the post-treatment examination, serum and 24-hour urine samples were taken from 17 *S. mansoni* infected patients at one, three and six weeks after chemotherapy.

Treatment of samples

The serum samples were stored frozen (−20°C) until use and treated before testing with TCA to remove interfering proteins and to dissociate immune complexes.[19]

The urine samples were kept frozen (−20°C) with 0.1% (w/v) sodium azide as a preservative until use. Before testing, aliquots of urine were treated with TCA, dialyzed, lyophilized and reconstituted as described by De Jonge and others, resulting in a 5-fold concentration of the original samples.[16]

The creatinine concentration of urine samples was determined within two days after collection by the Jaffe method,[20] using a 550 Express Clinical Chemistry Analyzer (Ciba Corning Diagnostics Corp., Oberlin, OH).

ELISA procedure

The ELISA technique for the quantitative determination of CAA was performed as described by Deelder and others, using the anti-CAA monoclonal antibody 120-1B10-A both as the capture antibody and as the alkaline phosphatase-conjugated second antibody.[15] The production of the mouse monoclonal antibody (IgG_1) directed against CAA has been described previously.[14, 15] In short, polyvinylchloride flat-bottomed microtitration plates (Flow, Irvine, UK) were coated with CAA mouse monoclonal antibody (ascitic fluid, diluted 1:1,000) and, after washing, blocked with bovine serum albumin. Plates were then washed and stored dry at −70°C until use. Urine and serum samples were tested in doubling dilutions. After washing, alkaline phosphatase-conjugated protein A-Sepharose (Pharmacia, Uppsala, Sweden)-purified, mouse monoclonal antibody 120-1B10-A was added to the wells. Following incubation, the plates were washed and incubated with *p*-nitrophenyl phosphate. Absorbance was read with a Titertek Multiskan MCC/340 MKII plate reader (Flow, Irvine, UK). The reciprocal value of the last sample dilution showing coloring above background level (mean absorbance of buffer controls plus 2 × standard deviation) was taken as the titer. Serum samples with a titer <4 were considered to be negative, samples with a titer ≥4 as positive. For urine samples this was <0.2 and ≥0.2 respectively. The lower detection limit was determined by titration of a solution of the TCA-soluble fraction of adult worm antigen (AWA-TCA).

Statistical methods

Since neither schistosome egg output, circulating antigen titers nor creatinine concentration showed normal distribution, nonparametric statistical methods were used to evaluate the results. Data were characterized by their medians and 90th percentile values. Spearman's rank correlation was used to calculate the concordance between data. The Friedman test and the Wilcoxon's signed ranks test were used to analyze data from urine collected at different time intervals, and to make the comparison of pretreatment and post-treatment data. The tests were run on an IBM PC/XT compatible computer using the $SPSS_{PC}$+/ statistical package (SPSS Inc., Chicago, US).

RESULTS

The fecal egg output of the 20 *S. mansoni*-infected patients ranged from 50 to 976 eggs per

TABLE 1

CAA titer and creatinine concentration in urine samples collected at four different periods of the day from 15 schistosomiasis patients

Group*** (n = 15)	Time of collection	CAA titer* creatinine concentration**			
		Minimum	Maximum	Median	90th percentile
Morning	08.00–12.00	0.1/0.3	51.2/2.5	25.6/1.8	6.4/0.6
Midday	12.00–16.00	0.1/0.3	25.6/1.0	12.8/0.9	3.2/0.7
Evening	16.00–20.00	0.1/0.2	51.2/1.3	12.8/1.1	3.2/0.5
Night	20.00–08.00	0.1/0.3	51.2/1.6	6.4/1.1	6.4/0.7

* Titers expressed as urine dilutions.
** Concentration expressed as mg/ml.
*** Group = 8 *S. mansoni*-infected patients and 7 patients infected with both *S. mansoni* and *S. haematobium*.

gram of feces (epg) (median 275 epg, 90th Percentile 875 epg). In patients with mixed infections, the fecal egg output ranged from 13 to 597 epg (median 156, 90th percentile 594 epg). Urine egg-counts ranged from 1 to 340 eggs/10 ml urine (median 13, 90th percentile 152 eggs/10 ml). No viable eggs could be demonstrated in the feces or urine of the 17 control subjects.

The lower detection limit of the ELISA for the quantitation of CAA was 0.8–1.56 ng AWA-TCA/ml. Of the 17 controls all the serum and urine samples had a titer of <4 and <0.2 respectively. The intra-assay imprecision for urine tests was 7.8% (n = 5), while the inter-assay imprecision was 11.7% (n = 3).

Table 1 summarizes CAA levels and creatinine concentrations of urine samples collected at four different time intervals during a 24-hour period. No significant difference in either CAA titer or creatinine concentration could be demonstrated (Friedman, n = 15, P = 0.54), even when the results were expressed as the corresponding ng AWA-TCA/mg creatinine (unpublished data). Subsequently, urine CAA titers of these patients were represented by the median of these four different collections.

Table 2 summarizes the results of testing serum and urine samples of the two patient groups. In 23 of the 28 patients (82%) serum CAA titers were ≥4, and in 25 patients (89%) urine CAA titers were ≥0.2.

Figure 1 shows the results of testing urine and serum samples of 17 patients infected with *S. mansoni* before, and one, three and six weeks after praziquantel treatment. CAA titers in urine (Fig. 1a) and serum (Fig. 1b) decreased significantly after one week (Wilcoxon, n = 13, $P<0.05$). No CAA was detectable in urine of eight patients (47%) at three weeks nor in urine of nine patients (69%) at six weeks, while all serum CAA titers became negative during the same period. Repeated parasitological examination showed one patient with viable eggs four weeks after treatment, but no eggs could be demonstrated two weeks later. No viable eggs were found for the other 14 patients three to six weeks after treatment; two patients were not examined.

The antigen level in urine showed a strong correlation with the level in serum, both before (n = 28, rho = 0.80, $P < 0.001$) and one week after treatment (n = 17, rho = 0.58, $P < 0.01$). A positive correlation was also found between CAA titers in serum and the number of parasite eggs in feces of patients infected with *S. mansoni* (n = 20, rho = 0.53, $P < 0.01$).

DISCUSSION

The demonstration of CAA in serum proved to have a strong diagnostic potential for schistosomiasis. This is because its day-to-day fluctuation during active infection was found to be

TABLE 2

CAA titer in serum and urine of 28 schistosomiasis patients

Group	n	CAA titer serum*/CAA titer urine*				
		% Se	Minimum	Maximum	Median	90th percentile
S. mansoni	20	85/90	2/0.1	4,096/51.2	128/3.2	1,024/25.6
Mixed**	8	75/87	2/0.1	2,048/51.2	192/4.8	2,048/6.4

Se = sensitivity; n = number of patients.
* Titers expressed as serum or urine dilutions.
** Patients infected with both *S. mansoni* and *S. haematobium*.

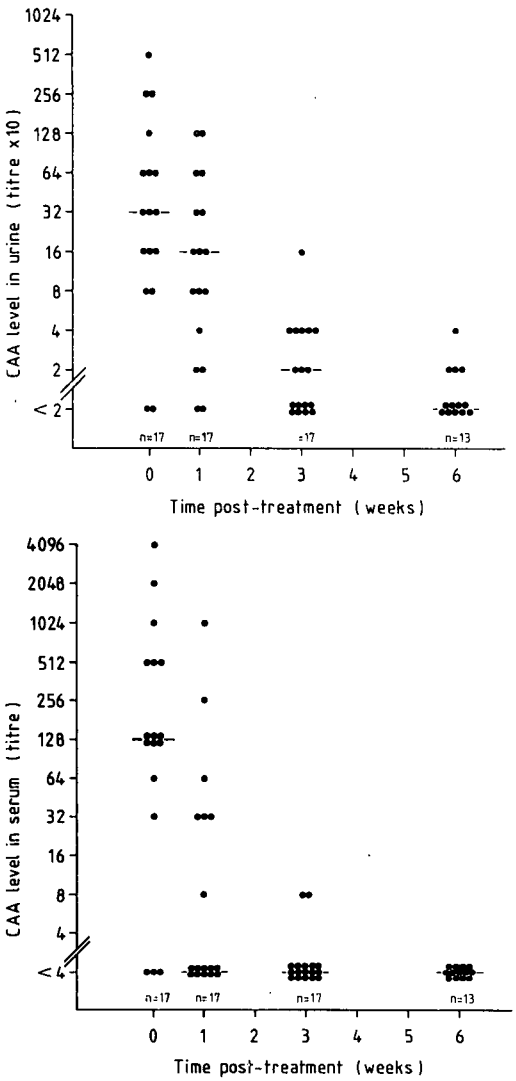

FIGURE 1. CAA titers in urine (1a) and serum (1b) samples from 17 patients infected with *S. mansoni*, before treatment and at one, three and six weeks after treatment with praziquantel (60) mg/kg, orally). —- , median; n = number of patients.

minimal, no false positive results could be shown and the level of CAA correlated with the intensity of infection.[2, 3, 15, 21] The presence of CAA could be detected in the serum of patients infected with *S. mansoni*,[1-3] *S. haematobium*,[1, 16] *S. japonicum* (N. De Jonge, unpublished data) and *S. intercalatum*.[22]

Following successful chemotherapy with praziquantel, this antigen disappears from the serum of *S. mansoni*-infected patients within about ten days.[3] Recently we have shown that quantitation of CAA in serum could be used to indicate efficacy of chemotherapy after treatment with either metrifonate, oxamniquine or praziquantel in patients infected with *S. mansoni* and *S. haematobium*.[23]

In mass population-control programs, however, the use of urine samples for non-invasive diagnosis of this systemic parasitic disease would be highly desirable. Although in *S. haematobium* infections urine samples can be evaluated by the reagent strip technique (dipstick) for the presence of blood or protein,[24] this method is not specific and cannot be used for other *Schistosoma* infections.

Several schistosome antigens have been demonstrated in the urine of patients. However the only other antigen which has been extensively characterized is the circulating cathodic antigen (CCA), also known as 'antigen M'.[25] The presence of CAA in the urine of patients infected with *S. mansoni* or *S. haematobium* was demonstrated only recently.[16] In that study the antigen was found in 81% of the intestinal schistosomiasis patients and in 97% of those with urinary schistosomiasis. A correlation with the serum CAA level and the fecal egg output was shown in patients with schistosomiasis mansoni.

Another polysaccharide antigen has recently been described by Ripert and others. Using a mouse monoclonal IgM-based inhibition hemagglutination assay, this antigen was demonstrated in the urine of 59% of 147 inhabitants of Cameroon, of whom 31% were actively passing *S. mansoni* eggs in their stool. Nine months after chemotherapy, 20% of the 97 subjects re-examined showed antigen in their urine, while only 2% were still voiding eggs.[5] No quantitative correlation was done.

In the current study urine and serum samples of Egyptian patients with *S. mansoni* or with mixed intestinal and urinary infections were tested before, and one, three and six weeks after treatment. No CAA could be demonstrated in urine samples from 17 Egyptians without an active *Schistosoma* infection which is consistent with previous results obtained for residents of The Netherlands.[16]

No significant difference in the level of CAA was found in urine samples collected at four intervals during a 24-hour period, spread over 5–12 days; this indicates both a low day-to-day fluctuation and that there is no circadian variability in the excretion of CAA. The urine sam-

ples did not vary significantly in their concentration when they were normalized against creatinine levels.

Before treatment, 85% of the 20 patients infected with *S. mansoni* showed CAA in serum and 90% in urine, compared to 75% and 88%, respectively, for the eight patients with mixed infection. The difference in sensitivity between serum and urine could be explained by the fact that the urine samples were tested at a higher concentration. A very strong correlation in CAA titer was found between urine and serum (rho = 0.80; $P < 0.001$). Consistent with previous results, we found a positive correlation between the number of *S. mansoni* eggs excreted in the feces and the CAA titers in serum.[15] No correlation was found between the egg load and urine CAA titers, even when the levels of CAA were corrected for the creatinine concentration.

Following chemotherapy (praziquantel, 60 mg/kg), a significant decrease in the CAA titer was found both in serum and urine after one week. Thereafter, the profile of CAA titers in urine continued to show a parallel but delayed decline to that of serum. All serum samples became negative three to six weeks after treatment. Low titers of CAA were still present in 53% of the urine samples at three weeks and in 31% at six weeks. This difference could be explained by the fact that the urine samples were tested at a concentration 20 times higher than for serum, and by the retention and slow release of antigen from kidney tissue. Immune complexes containing CAA and CCA have been shown to be present in the glomeruli of *S. mansoni*-infected mice, hamsters and humans.[26, 27] Six to eight months after chemotherapy no difference could be demonstrated in the intensity of CAA or CCA in kidney biopsies of 10 of the 15 patients studied, using an indirect immunofluorescence assay.[27]

In conclusion, the present study demonstrates that the CAA urine assay could be used, independent of the time of sample collection, as a sensitive and non-invasive method to diagnose schistosomiasis and to monitor the efficacy of chemotherapy. Further development of the assay will require improvement of the method of sample concentration and determination of the minimum volume of urine needed for appropriate quantitation of CAA.

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Chapter 2.4

Circulating cathodic antigen levels in serum and urine of schistosomiasis patients before and after chemotherapy with praziquantel.

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Short Report

Circulating cathodic antigen levels in serum and urine of schistosomiasis patients before and after chemotherapy with praziquantel

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Detection of circulating anodic antigen (CAA) and circulating cathodic antigen (CCA) in serum and urine has been shown to be a valuable alternative to egg counts for the diagnosis of schistosome infections. The levels of CAA and CCA were found to be highly specific and sensitive markers for the intensity of infection (DEELDER *et al.*, 1989; DE JONGE *et al.*, 1990). Circulating antigen assay is also useful in monitoring therapeutic intervention. Following successful chemotherapy with praziquantel, CAA disappeared from serum within about 10 d (DE JONGE *et al.*, 1989), and from urine within several weeks (VAN LIESHOUT *et al.*, 1991). No post-treatment study of CCA has been performed.

The present study was designed to evaluate the kinetics of CCA after chemotherapy. All individuals included in this study gave informed consent. Serum and urine samples were collected from 17 Egyptian patients with *Schistosoma mansoni* infection, before, and 1, 3, and 6 weeks after, treatment with 60 mg/kg praziquantel (Biltricide®), administered in 3 doses of 20 mg/kg orally at 4–6 h intervals. No urine sample was collected from one individual. All patients were males in the age range 11–49 years (median 17 years) and remained in hospital until 3–6 weeks after chemotherapy. Before treatment the faecal *S. mansoni* egg output ranged from 50 to 976 eggs/g of faeces (median 260 eggs/g). The CCA level was measured in serum and urine by a monoclonal antibody-based enzyme-linked immunosorbent assay (ELISA) (DE JONGE *et al.*, 1990). Serum and urine samples were titrated (dilutions ranging from 1:4 to 1:4096) and considered to be positive for CCA if the reciprocal titres were ≥ 8 and ≥ 16, respectively, based on previous results with control sera from uninfected individuals in an endemic area (VAN LIESHOUT *et al.*, 1992). The lower detection limit of this ELISA was 3·9–7·8 ng/mL of the trichloroacetic acid-soluble fraction of adult worm antigen (AWA-TCA), which contains approximately 3% CCA (G. J. Van Dam, personal communication).

Serum and urine CCA levels before and after treatment are shown in the Figure. Before chemotherapy, all serum samples and 81% of the urine samples were positive. After one week, CCA titres in serum and urine decreased significantly (Wilcoxon's test, $P<0{\cdot}001$, $n=17$ and 16). All serum and most (92%) urine titres became negative 3–6 weeks after treatment. Overall, the decline of CCA levels in serum and urine showed a profile similar to serum CAA in this group (VAN LIESHOUT *et al.*, 1991). In 14 patients no viable eggs were found at repeated parasitological examination 3–6 weeks after treatment; data could not be obtained from 3 patients.

A significant correlation was found between the number of parasite eggs in faeces and the CCA level in serum before treatment (Spearman's $\rho=0{\cdot}71$, $P=0{\cdot}001$, $n=17$), and between serum and urine CCA level before and after treatment (Spearman's $\rho=0{\cdot}50$, $P<0{\cdot}001$, $n=61$). No other significant correlation was found.

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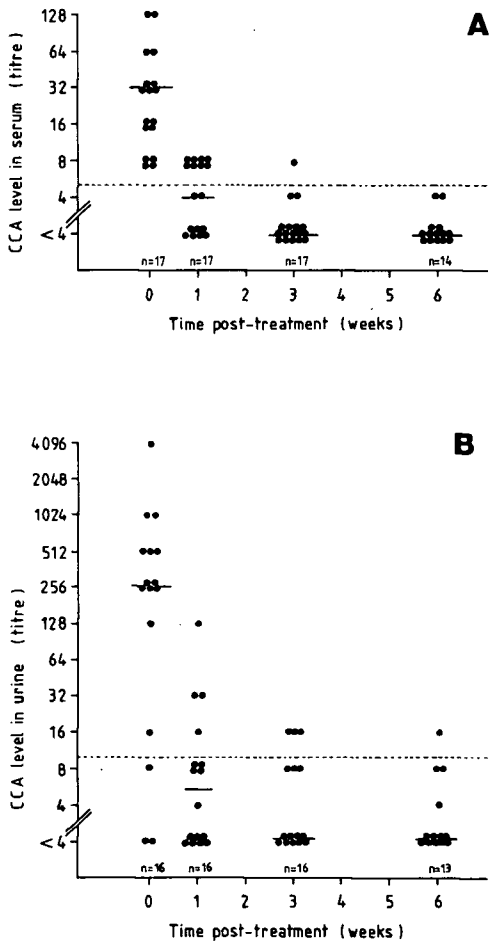

Figure. Reciprocal CCA titres in serum (A) and urine (B) samples of patients with schistosomiasis mansoni before treatment and at one, three and six weeks after treatment with praziquantel (60 mg/kg, orally). Horizontal lines indicate median values; broken line shows negative/positive cut-off value; n=number of patients.

In conclusion, this study demonstrated that the CCA assay can be used with both serum and urine as a sensitive method to monitor the efficacy of chemotherapy. The use of urine samples only would be highly desirable for mass population-screening programmes because it is non-invasive. The level of CCA in urine does not show any circadian variability (unpublished data), which is consistent with previous results obtained for CAA (VAN LIESHOUT *et al.*, 1991). In addition, the CCA urine assay has the advantage, compared to the CAA urine assay, that it does not require a time consuming concentration procedure.

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Chapter 2.5

Monitoring the efficacy of different doses of praziquantel by quantification of circulating antigens in serum and urine of schistosomiasis patients.

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Monitoring the efficacy of different doses of praziquantel by quantification of circulating antigens in serum and urine of schistosomiasis patients

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SUMMARY

We evaluated the quantitation of two schistosome circulating antigens in serum and urine as a tool for the assessment of the efficacy of praziquantel dosage regimens (40 versus 60 mg/kgbw). In addition we compared the efficacy of two different brands of praziquantel (Biltricide® and Distocide®), given at the same dosage (40 mg/kgbw). Thirty five Egyptian hospitalized schistosomiasis mansoni patients participated in this study. Thirteen patients (Group 1) received 60 mg/kgbw Biltricide®, administered in 3 oral doses of 20 mg in one day; 22 individuals (Group 2) were treated with 40 mg/kgbw (12 Biltricide®, 10 Distocide®), given in one oral dose. Circulating anodic antigen (CAA) and circulating cathodic antigen (CCA) were quantitated by monoclonal antibody-based ELISA's before, and 1, 3 and 6 weeks after chemotherapy. Before treatment, all patients were positive for at least one of the circulating antigen assays. Three to six weeks after treatment significantly more patients were found to be negative in Group 1 compared to Group 2 ($X^2 = 7{\cdot}13$, $P = 0{\cdot}008$, $n = 35$). Also the levels of CCA and CAA in serum and of CCA in urine were found to be significantly higher in Group 2 (Mann-Whitney $U < 85$, $P < 0{\cdot}05$, $n = 35$). These results were confirmed by parasitological data. No differences were found between treatment with Biltricide® or Distocide®. Our results indicate that praziquantel treatment of schistosomiasis with 60 mg/kgbw divided in 3 doses results in a higher cure rate compared to 40 mg/kgbw as a single dose, and provide further evidence for the use of the CAA and CCA assays as a sensitive method to monitor the efficacy of chemotherapy, particularly when circulating antigen assays are combined by parallel testing.

Key words: schistosomiasis, praziquantel, Biltricide®, Distocide®, drug efficacy, circulating anodic antigen (CAA), circulating cathodic antigen (CCA).

INTRODUCTION

Praziquantel is currently the drug of choice for the treatment of *Schistosoma* infections in humans (reviewed by Webbe, 1987; King & Mahmoud, 1989; Shekhar, 1991). A single oral dose of 40 mg/kg of bodyweight (kgbw) is the standard recommended for both *S. mansoni* and *S. haematobium* infections. The drug was initially introduced as Biltricide® by the German-Swiss company Bayer-Merck, but is now also manufactured as Distocide® by the Korean company Shin-Poong Pharmaceutical.

Although cure rates of 70–100% are generally stated, lower cure rates have also been reported (Polderman, Gryseels & De Caluwé, 1988; El-Masry, Bassily & Farid, 1988; Stelma *et al.* 1992). No differences in efficacy were found between Distocide® and Biltricide® (Homeida *et al.* 1989). Improved cure rates have been described if praziquantel was administered in split doses given up to 6 h apart (El-Masry *et al.* 1988). A difficulty in determining cure rates is the problem of differentiating between failure of treatment and reinfection. Also, cure rates are dependent on the sensitivity of the diagnostic technique used because, particularly after treatment, light infections can easily be missed (Polderman *et al.* 1988; De Vlas & Gryseels, 1992).

Quantitation of circulating anodic antigen (CAA) and circulating cathodic antigen (CCA) in serum and urine has increasingly been recognized as a valuable and feasible approach for the diagnosis of *Schistosoma* infections. The levels of these adult worm gut-associated antigens correlate well with the intensity of infection, and both CAA and CCA have been reported to disappear rapidly from serum and urine after successful treatment (Deelder *et al.* 1989; De Jonge *et al.* 1988, 1989*a*, *b*; 1990*a*; Barsoum *et al.* 1991; Van Lieshout *et al.* 1991, 1993; Van 't Wout *et al.* 1992).

In a previous study, we improved diagnostic

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performance by parallel testing for CAA and CCA in serum and urine of 68 Egyptian schistosomiasis patients, before chemotherapy (Van Lieshout *et al.* 1992). The purpose of the present study was to evaluate the use of the circulating antigen assay for monitoring the efficacy of different treatment protocols. Therefore we retrospectively analysed both CAA and CCA levels in serum and urine from 35 of these patients, following treatment with either 40 or 60 mg/kgbw praziquantel. In addition, we also compared circulating antigen levels between those who received Distocide® and those who received Biltricide®, both in one dose of 40 mg/kgbw.

MATERIALS AND METHODS

Study population

Serum and urine samples of 35 Egyptian hospitalized patients were analysed for their CAA and CCA levels, before and after chemotherapy. On the day of admission all subjects showed live *S. mansoni* eggs in their stools; two cases also showed *S. haematobium* eggs in their urine (7 and 11 eggs/10 ml). All individuals were part of a previous study on improvement of diagnostic performance by parallel testing of CAA and CCA in serum and urine of 68 male schistosomiasis patients (van Lieshout *et al.* 1992). Patients were selected for post-treatment analysis if they had been followed up for at least 6 weeks after chemotherapy.

Patients were treated with two different doses of praziquantel, depending on the current protocol at the moment of hospitalization. Thirteen individuals (Group 1) received 60 mg/kgbw praziquantel (Biltricide®, manufactured by Alexandria Co. for Pharmaceuticals and Chem. Ind., Alexandria, Egypt, under licence of Bayer Leverkusen, Germany) administered in 3 oral doses of 20 mg/kgbw, given at 4–6 h intervals. The other 22 subjects (Group 2) received 40 mg/kgbw, given in 1 oral dose. The individuals of Group 2 were part of a larger patient group in which the side-effects of two different brands of praziquantel were examined in a double-blind randomized study. For this reason 12 individuals received Biltricide®, and the other 10 Distocide® (produced by EIPICO, Cairo, Egypt, under licence of Shin-Poong, Korea). The two patients with mixed *S. mansoni* and *S. haematobium* infection participated in Group 2, one received Biltricide®, the other Distocide®.

All individuals included in this study participated after informed consent was obtained.

Parasitological examination

Faecal egg excretion was determined by calculating the mean of 3 consecutive, duplicate, Kato-Katz stool examinations, starting on the day of hospitalization (Katz, Chaves & Pellegrino, 1972). Urinary egg excretion was determined by 2 consecutive 10 ml Bell examinations (Bell, 1967). A first parasitological follow-up was done 3–7 and a second 6–12 weeks post-chemotherapy. Each consisted of duplicate Kato slides on 2 consecutive days. The viability of the eggs was checked at every examination.

Collection and pre-treatment of serum and urine samples

Serum and urine samples were taken before and 1, 3 and 6 weeks after chemotherapy and were stored at −20 °C until use. All samples were pre-treated with trichloroacetic acid (TCA) to remove interfering proteins and to dissociate immune complexes (De Jonge, Fillié & Deelder, 1987). Samples were tested starting at a 4-fold dilution, except urine samples tested for CAA, which were dialysed, lyophilized and reconstituted in the assay buffer resulting in a 5-fold concentration of the original samples (De Jonge *et al.* 1989*b*).

Determination of circulating antigens

Both CAA and CCA were quantitated by a mouse monoclonal antibody-based sandwich ELISA (Deelder *et al.* 1989; De Jonge *et al.* 1990*a*), with some minor modifications (van Lieshout *et al.* 1992). The lower detection limit of the CAA ELISA was 0·8–1·6 ng/ml of the TCA-soluble fraction of adult worm antigen (AWA-TCA). For the CCA ELISA this was 3·9–7·8 ng AWA-TCA/ml. AWA-TCA contains approximately 3% (w/w) CAA and 3% (w/w) CCA (Van Dam *et al.* 1993).

Urine and serum samples were tested in doubling dilutions. The reciprocal value of the last sample dilution showing colouring above background level (mean absorbance plus 2 × standard deviation of buffer controls) was taken as the titre. Based on endemic controls, serum samples were considered to be negative for CAA and CCA if their titres were < 4 and < 8, respectively. For urine the negative titres were $< 0{\cdot}2$ and < 16, respectively (De Jonge *et al.* 1989*b*; van Lieshout *et al.* 1992). Each sample was assayed once and single-blinded.

Data analysis

To describe and evaluate the results, non-parametric statistical methods were used, because circulating antigen titres and schistosome egg output did not show a continuous or normal distribution. Data were characterized by their ranges and medians. Spearman's rank correlation test was used to calculate concordance, the Mann-Whitney test to calculate differences between groups and, the Wilcoxon's signed rank test to compare pre- and post-

Table 1. Pre-treatment characteristics of the total group of schistosomiasis patients as well as of Group 1 and Group 2 (assigned according to their praziquantel treatment schedule)

	All patients ($n = 35$)	Group 1§ ($n = 13$)	Group 2 ($n = 22$)	Significance level P
Age*	10–49 (16)	11–49 (17)	10–26 (15)	0·02
Epg†	10–1213 (183)	50–976 (290)	10–1213 (110)	0·09
CAA‡				
Serum	2–4096 (110/89%)	2–4096 (128/85%)	2–2048 (96/90%)	0·17
Urine	0·1–102·4 (3·0/86%)	0·1–25·6 (3·2/95%)	0·1–102·4 (2·4/82%)	0·43
CCA				
Serum	2–128 (32/97%)	8–128 (32/100%)	2–128 (32/95%)	0·73
Urine	2–4096 (410/86%)	2–2048 (256/77%)	2–4096 (512/90%)	0·29

* Minimum–maximum (median) of age (years).
† Minimum–maximum (median) of *S. mansoni* eggs/gram faeces (epg). Two individuals of Group 2 also excreted *S. haematobium* eggs, 7 and 11 eggs/10 ml of urine, respectively.
‡ Minimum–maximum (median/% positive) of: circulating anodic antigen (CAA) and circulating cathodic antigen (CCA) in serum and urine (expressed in titres).
§ Following pre-treatment examination, praziquantel was given to all patients. Group 1 received 3 doses of 20 mg/kgbw each at 4–6 h intervals, Group 2 a single dose of 40 mg/kgbw.

treatment data. The Chi-square analysis (including Yates correction) or the Fisher's exact test were used to evaluate assay properties.

RESULTS

Pre-treatment data

Table 1 summarizes the age, faecal egg counts and circulating antigen levels of the two patient groups before chemotherapy. Group 1 was slightly older. No significant differences were found in egg output or antigen levels. A significant correlation was found between the number of parasite eggs in the faeces and the level of circulating antigen for each assay (Spearman's rho ranging from 0·49 to 0·69, $P < 0·003$, $n = 35$). In Group 2, no differences were found between those who received Distocide® and those who received Biltricide®, for all variables mentioned (Mann-Whitney $U > 38$, $P > 0·16$, $n = 22$).

Egg excretion after treatment

Following chemotherapy, no viable eggs could be demonstrated in stool or urine of 11 persons of Group 1, treated with 60 mg/kg praziquantel. One patient showed a mean faecal egg output of 10 eggs/g faeces (epg) at 4 weeks, but was negative at 6 weeks; another showed no eggs at 4 weeks, but was positive in the hatching test at 12 weeks. Thus, the parasitological curve rate in Group 1 was 85%, with a reduction in egg numbers in those still positive of 98%.

In Group 2, treated with 40 mg/kgbw, 12 patients were found egg negative, which corresponds to a cure rate of 55%. The other 10 subjects showed viable *S. mansoni* eggs in their faeces. Eight of them were positive at the first follow-up after the treatment, with egg counts of 5–140 epg (median 23 epg). All 10 individuals excreted viable eggs at the second follow-up, with egg counts between 0 (only hatching test positive) and 120 epg (median 23 epg). In one individual the egg output increased from 20 epg before treatment, to 140 epg at 4 weeks and 35 epg at 6 weeks post-chemotherapy. In the others the egg count reduction ranged from 40 to 99% (median 88%). No viable *S. haematobium* eggs were found during the follow-up period. For those treated with Distocide® and Biltricide®, no eggs could be demonstrated in 50% and 58% of the subjects, respectively.

Circulating antigen level after treatment

For both patient groups, the CAA serum level decreased significantly within 1 week after chemotherapy (Wilcoxon, $P \leq 0·005$). However, while all subjects of Group 1 became negative for this antigen during the follow-up period (Fig. 1A), 9 patients (41%) of Group 2 still showed a low,

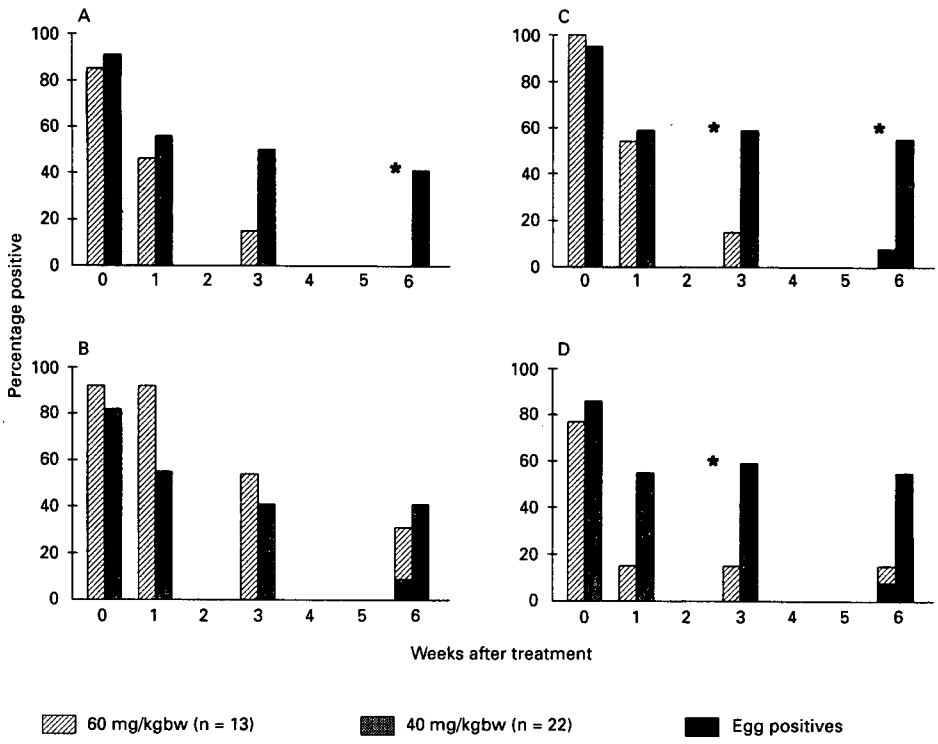

Fig. 1. Percentages of patients positive for circulating antigen before and after treatment with 60 mg/kgbw (Group 1, $n = 13$) or 40 mg/kgbw (Group 2, $n = 22$) praziquantel. (A) Circulating anodic antigen (CAA) in serum; (B) CAA in urine; (C) circulating cathodic antigen (CCA) in serum; (D) CCA in urine. The black portions of the bars at week 6 represent the percentages of patients who were also positive for the parasitological examination during the follow-up period (* = $X^2 > 3{\cdot}8$, $P < 0{\cdot}05$).

but clearly positive titre at week 6 (titres in the range of 8–32, median 32). Five of them also excreted viable eggs. The difference between Group 1 and 2 was significant at week 6, both in the percentage of positives ($X^2 = 5{\cdot}2$, $P = 0{\cdot}02$) and in the level of CAA (Mann-Whitney U = 85, $P = 0{\cdot}009$).

As can be seen in Fig. 1 B, the kinetics of CAA in urine following chemotherapy were somewhat different from those in serum. Although the concentration levels of CAA decreased significantly within 1 week for both groups (Wilcoxon, $P = 0{\cdot}05$ and $P = 0{\cdot}001$), the number of positives was still high. Six weeks after treatment, CAA could still be demonstrated in 4 individuals (31 %) of Group 1, showing low titres (ranging from 0·2 to 0·4, median 0·2). One of these four also excreted viable eggs. In Group 2, 9 patients (41 %) were still positive at this point (titre from 0·2 to 1·6; median 0·4), with 5 of these 9 subjects excreting eggs. The difference between Group 1 and 2 was not statistically significant during the follow-up, neither in percentage of positives ($X^2 = 0{\cdot}06$, $P = 0{\cdot}81$), nor in the CAA level (Mann-Whitney U = 119, $P = 0{\cdot}33$).

CCA concentration in serum decreased significantly within 1 week for both groups (Wilcoxon, $P < 0{\cdot}002$). All patients treated with 60 mg became negative, except 1 (titre of 16) (Fig. 1 C). This single patient also excreted viable eggs (10 epg). In Group 2, 12 individuals (55 %) were still positive after 6 weeks (titre from 8 to 64; median of 16), with 8 of them also excreting eggs. Starting at week 3, a significant difference was found between the number of positives in the two groups ($X^2 > 4{\cdot}7$, $P < 0{\cdot}03$), and in the level of antigen (Mann-Whitney U < 88, $P < 0{\cdot}04$).

As can be seen in Fig. 1 D, the kinetics of CCA in urine look similar to those in serum. The concentration of CCA in urine decreased significantly within 1 week after chemotherapy for both groups (Wilcoxon, $P < 0{\cdot}003$). Only 2 subjects of Group 1 were still positive at the end (titre 16 and 128), 1 of them excreting eggs. In Group 2, 12 patients (55 %) were still positive 6 weeks after chemotherapy (titre 16–128; median 64), of whom 6 showed viable eggs. At week 3, a significant difference was found between the number of positives in the two groups ($X^2 = 4{\cdot}7$, $P = 0{\cdot}03$), and at week 3 and week 6 in the level of antigen (Mann-Whitney U < 73, $P < 0{\cdot}01$).

In Group 2, no differences were found during the follow-up period for any of the circulating antigen

Table 2. Efficacy of treatment (expressed as number and percentage of individuals found to be negative) based on parasitological examination and circulating antigen determination in schistosomiasis patients treated with either 60 mg/kgbw praziquantel (Group 1) or 40 mg/kgbw praziquantel (Group 2)

	Group 1 ($n = 13$)	Group 2 ($n = 22$)	Significance level P
Parasitological examination*	11 (85%)	12 (55%)	0·15
Circulating antigen determination†	11 (85%)	7 (32%)	< 0·01
Overall	10 (77%)	6 (27%)	0·01

* Demonstration of viable eggs 3–12 weeks after treatment (see also text).
† Demonstration of CAA in serum and CCA in serum and urine, 6 weeks after treatment.

assays between those who received Distocide® and those who received Biltricide® ($X^2 < 1·5$, $P > 0·22$ and Mann-Whitney U > 40, $P > 0·12$).

Combined circulating antigen data

Diagnostic values could be increased when the data of CAA levels in serum and CCA levels in serum and urine were combined in a parallel fashion. Patients were considered to be positive for circulating antigens, if positive titres could be demonstrated in at least 1 of these 3 immunodiagnostic assays. Before chemotherapy all subjects were found to be positive. During the follow-up period, all individuals of Group 1, except 2, became totally negative for these circulating antigens, with 1 of the 2 excreting viable eggs. In Group 2, 15 patients were still positive 6 weeks after praziquantel treatment, of whom 9 showed viable eggs. The difference between Group 1 and 2 in the number of positives was significant, starting at week 3 ($X^2 > 3·8$, $P < 0·05$).

Table 2 summarizes the efficacy of treatment based on either parasitological or circulating antigen diagnosis. Taking Group 1 and Group 2 together, 16 individuals were found to be completely cured based on both diagnostic methods. The pre-treatment intensity of infection of these 16 subjects was not found to be significantly lower than that of the non-cured individuals (calculated for both egg output and circulating antigen level, Mann-Whitney U > 106, $P > 0·1$, $n = 35$).

In each group there was one individual who still passed viable eggs but became negative for the circulating antigen assays within 6 weeks. In Group 1, this individual was only parasitologically positive in the hatching assay at week 12. Serum and urine samples collected from this person at week 12 were found to be clearly positive for circulating antigens. The individual of Group 2 was hatching positive at weeks 6 and 10 (Kato 20 and 15 epg, respectively). From this person, no more serum or urine samples were taken after week 6.

All patients with parasitologically proven failure of cure were re-treated with 60 mg/kgbw praziquantel divided in 3 doses. Four of them could be followed for a few more weeks. All 4 became negative for CAA and CCA in serum and urine and at the parasitological examination when re-tested 1–4 weeks later.

DISCUSSION

At present, the focus in schistosomiasis control programmes is on the use of specific chemotherapy for the reduction of morbidity by decreasing intensity of infections rather than on the killing of all parasites (Wilkins, 1989). However, the assumption that significant morbidity is only present in individuals with high egg counts is still controversial (Gryseels & Polderman, 1991). Undetected and untreated light infections may also be important for the transmission following selective chemotherapy. To diagnose light infections or individual failures of treatment, standard egg counts have to be repeated several times to avoid a significant underestimation (De Vlas & Gryseels, 1992).

Detection of circulating antigens has been shown to be a sensitive and highly specific alternative for the diagnosis of an active *Schistosoma* infection (De Jonge *et al.* 1988). The concentration of circulating antigen shows less day-to-day fluctuation than egg counts (De Jonge *et al.* 1989*a*), and could therefore be measured in one single serum or urine sample. Quantification of circulating antigens is also a promising technique to differentiate between persistent and newly acquired infections, because circulating antigens are cleared within several days to a few weeks following successful treatment (De Jonge *et al.* 1989*a*; Barsoum *et al.* 1991; van Lieshout *et al.* 1991, 1993; Van 't Wout *et al.* 1992). Although most post-treatment studies have concentrated on the well-characterized adult worm gut-associated circulating antigens CAA and CCA, other schistosome circulating antigens have also been reported to decrease following chemotherapy, including both adult worm and egg antigens (Madwar,

Hassan & Strickland, 1988; Ripert *et al.* 1989; Hassan, Badawi & Strand, 1992).

Both CAA and CCA are genus specific, they have been demonstrated in the serum and urine of patients infected with *S. mansoni*, *S. haematobium*, *S. japonicum* and *S. intercalatum*, and in most of these studies the antigen levels correlated significantly with the intensity of infection (De Jonge *et al.* 1988, 1989*b*; Van 't Wout *et al.* 1992; Kremsner *et al.* 1993).

Circulating antigen detection was applied to compare different anti-schistosomal treatment protocols in a study by De Jonge *et al.* (1990*b*). In that study, CAA serum levels were determined in a group of Sudanese with mixed *S. mansoni* and *S. haematobium* infections, 1 and 5 months after treatment with a placebo, praziquantel, oxamniquine or metrifonate. While the placebo and the metrifonate did not result in a reduction of the CAA level, CAA was below the detection level 1 month after treatment with praziquantel. Treatment with oxamniquine resulted in a partial reduction of the serum antigen level (De Jonge *et al.* 1990*b*).

In the present study, subjects were not randomized but treated according to the current protocol at the moment of hospitalization. Before chemotherapy, no significant differences were found between the two patient groups in respect to intensity of infection, as reflected by egg output and circulating antigen level. Group 2, patients treated with 40 mg/kgbw, were somewhat younger. This could be of importance, since a lower cure rate has been described in children than in adults (Polderman *et al.* 1988).

Following treatment, a higher percentage of parasitologically negative individuals was found in Group 1 compared to Group 2, although these differences were not found to be statistically significant. Compared to most other published data the cure rates found here are relatively low, which could be explained by the intensive method of parasitological examination. On the other hand, in those not completely cured we found a median reduction of egg output comparable to other studies (Katz, Rocha & Chaves, 1981; Massoud, El Kholy & Anwar, 1984; El-Masry *et al.* 1988).

Of the single circulating antigen assays, the serum CCA assay showed the best performance in pointing out the failures of treatment, taking the parasitological examination as the gold standard. In concordance with previous data, the levels of CAA in urine decreased more slowly than those of other antigens, which makes it less suitable for monitoring the efficacy of chemotherapy (van Lieshout *et al.* 1991). The slow decrease in urine CAA concentration could be explained by retention and slow release of this antigen from kidney tissue. Therefore the CAA urine assay was not included in the combined analysis.

The most pronounced difference between Group 1 and Group 2 was obtained if data were combined in a parallel fashion, i.e., an individual was considered to have an active infection when showing a positive titre for either CAA in serum or CCA in serum or urine. In that case, 85% of the subjects of Group 1 were found to be completely negative within 6 weeks, compared to 32% in Group 2. Those remaining positive showed decreased antigen levels.

No differences were found in post-treatment data for individuals receiving Biltricide® or Distocide®. Although these data were obtained for a small number of subjects, they confirmed the finding of an extensive comparative epidemiological and pharmacological study in which it was demonstrated that Distocide® is as effective as Biltricide®, with lesser side-effects, probably due to its lower plasma levels and longer duration of action (Homeida *et al.* 1989; Mandour *et al.* 1990).

In summary, our results demonstrated that higher cure rates can be obtained after treatment with 60 mg praziquantel given in 3 oral doses in 1 day, than after a single treatment with 40 mg/kgbw. Also, these results confirm that detection of circulating antigens can be used as a sensitive method to monitor the efficacy of chemotherapy, and that diagnostic performance is improved by parallel testing for more than one antigen.

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Chapter 2.6

Immunodiagnosis of schistosomiasis mansoni in a low endemic area in Surinam by determination of the circulating antigens CAA and CCA.

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Immunodiagnosis of schistosomiasis mansoni in a low endemic area in Surinam by determination of the circulating antigens CAA and CCA

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We evaluated the applicability of circulating antigen detection in serum and urine for the diagnosis of *Schistosoma* infections in a low endemic area. In total 389 individuals from Saramacca (Surinam) participated in the survey. Stool samples were examined using the Kato method, while circulating anodic antigen (CAA) and circulating cathodic antigen (CCA) were determined by highly specific monoclonal antibody-based ELISA's. Also schistosome specific IgM antibodies were measured by the indirect immunofluorescence assay, but the diagnostic performance of this test was found to be poor in this population. *S. mansoni* eggs were found in 29% of the examined cases, while CAA and CCA could be demonstrated in 23% and 17% of the serum samples and in 3% and 28% of the urine samples, respectively. Forty three percent of the study population was positive in at least one of these diagnostic assays, indicating that each individual test misses a substantial part of the subjects with an active infection. In most positive cases, intensities of infection were very low. As 204 individuals participated in all screening assays, diagnostic performance of each test was evaluated in this sub-population. The highest sensitivities were achieved with the urine-CCA assay and the parasitological examination, detecting 59 and 58 out of the 107 cases with an active infection, respectively. The serum-CAA assay detected 47 positive cases. Our results demonstrate that determination of circulating antigens, especially CCA in urine and CAA in serum, provides information additional to the parasitological examination, for the assessment of prevalence and intensity of *Schistosoma* infection in low endemic areas.

Key words: *Schistosoma mansoni*; Circulating anodic antigen; Circulating cathodic antigen; Immunodiagnosis; Low endemic area

1. Introduction

In Surinam, intestinal schistosomiasis due to *Schistosoma mansoni* infection is endemic in the coastal areas, where shell ridges form an ideal habitat for the intermediate host, *Biomphalaria glabrata*. Previous epidemiological studies showed that both prevalence and intensity of infection were not high in this area. Surveys done in the district of Saramacca revealed prevalences of 20–28% (Van der Kuyp, 1969; Deelder et al., 1980a). However, these studies were based on stool examination,

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which has the disadvantage that light infections can be easily missed (Teesdale et al., 1985; De Vlas and Gryseels, 1992).

Alternatively, detection of specific antibodies has shown to be a highly sensitive diagnostic method, although generally no correlations are found between antibody levels and intensities of infection. Besides, the presence of specific antibodies allows in general no differentiation between current and past infection (Mott and Dixon, 1982; Toswill and Ridley, 1986; Watt et al., 1986).

In recent years, it has been shown that determination of specific circulating antigens in serum or urine is a valuable diagnostical tool to discriminate individuals with an active *Schistosoma* infection from persons with a past or no infection (Abdel-Hafez et al., 1983; Feldmeier et al., 1986; De Jonge et al., 1988). Several circulating antigens have been described, but most studies have concentrated on the quantification of two glycoconjugates named circulating anodic antigen (CAA) and circulating cathodic antigen (CCA), both associated with the gut of the adult worm (Nash et al., 1977; Deelder et al., 1980b; Carlier et al., 1980). The monoclonal antibody based ELISA's, which demonstrate these antigens in serum or urine, show a specificity of at least 98%, with sensitivities ranging from approximately 70 to 100% (Deelder et al., 1989a; De Jonge et al., 1988, 1989b, 1990; Krijger et al., 1994). Most of the CAA and CCA concentrations found in schistosomiasis patients are clearly distinguishable from the cut-off levels of the assays. Even so, antigen levels correlate well with the intensity of infection, and following successful treatment these antigens are rapidly cleared from the circulation (De Jonge et al., 1988, 1989a,b; Van Lieshout et al., 1992, 1994).

Measurements of CAA and CCA as means of diagnosing *S. mansoni* infections, have been studied extensively for several study populations in Africa, mainly in areas with high prevalences and intensities of infection. (De Jonge et al., 1988, 1989a, 1989b; Van Lieshout et al., 1992). However, little data have been collected from schistosomiasis patients in low endemic areas. In a study of Feldmeier et al. (1986), sera of 28 schistosomiasis patients from Brazil were tested for the presence of CAA and CCA, applying assays not yet as sensitive as the ones described more recently. The patients showed a median egg output of 20 eggs per gram of faeces and only 11% and 32% were found to be positive for CAA and CCA, respectively.

The aim of this study was to determine the applicability of circulating antigen determination for epidemiological studies in areas with low prevalences of schistosomiasis mansoni. Therefore CAA and CCA levels were determined in serum and urine samples collected during a survey at a village in the district of Saramacca in Surinam, and compared with the faecal egg excretion and specific antibody levels.

2. Patients and methods

2.1. Area and population

The study took place in the village of Catharina Sophia in the district of Saramacca (Surinam), 50 km to the west of the capital Paramaribo. The total population of Catharina Sophia consists of approximately 500 inhabitants, most of which are of Javanese origin; agriculture is the principle means of existence. Informed consent was obtained from all individuals who participated in this study. Children younger

than 1 year were left out of the study. Persons who showed an active *S. mansoni* infection, were treated with a standard dose of praziquantel.

2.2. Parasitological examination

Stool samples were collected twice, with three weeks interval. Faecal egg excretion was determined by calculating the mean of two duplicate 25 mg Kato-smears (Katz et al., 1972).

2.3. Collection and treatment of blood and urine samples

Blood samples were taken by venipuncture, 4–6 weeks after the first stool collection. Serum was stored at −20°C, until use. Urine samples were collected at the same time as the second stool sample and also stored at −20°C.

All samples were pretreated with trichloro-acetic acid (TCA) to remove interfering proteins and to dissociate immune complexes (De Jonge et al., 1987). Samples were tested starting at a 4-fold dilution, except for urine samples tested for CAA, which were dialysed, lyophilised and reconstituted in buffer, resulting in a five-fold increase of the original concentration (De Jonge et al., 1989b).

2.4. Circulating antigen determination

Levels of CAA and CCA in serum and urine were determined by monoclonal antibody-based enzyme immunoassays as described previously (Deelder et al., 1989a; De Jonge et al., 1990). The lower detection limit of the CAA ELISA was 1–4 ng of the TCA-soluble fraction of adult worm antigen (AWA-TCA) per ml. For the CCA ELISA this was 2–8 ng AWA-TCA per ml. AWA-TCA contains approximately 3% (w/w) CAA and 3% (w/w) CCA (Van Dam et al., 1993).

All samples were tested in two-fold dilution series, whereby the reciprocal value of the last sample dilution showing colouring above background level, was taken as the titre. Based on previous studies, serum and urine samples were considered to be positive for CAA, and serum samples for CCA, when showing a titre >2, >0.8, and >4, respectively (Krijger et al., 1994). Due to a slightly different pretreatment procedure for the urine-CCA assay, cut-off levels were determined again for this test. Seventy urine samples of healthy Dutch individuals, who had no history of visiting an area where schistosomiasis is endemic, were pretreated similar to the Surinam urine samples. As all of them showed titres lower than 32, urine samples were considered to be positive for CCA when showing a titre >16.

2.5. Detection of specific antibodies

IgM antibodies were determined by an immunofluorescence assay (IFA) as described by Nash (1978). Antibodies demonstrated by this technique are mainly directed against CCA (Deelder et al., 1989b). Serum samples were tested in a doubling dilution series from 1/16 onward on sections of Rossman's fixed adult *S. mansoni* worms. Titres ≥ 16 were considered to be positive (Deelder et al., 1989c).

2.6. Data analysis

Since none of the data showed a continuous or normal distribution, non-parametric statistical methods were used to describe and evaluate the results. Data were characterized by their ranges and median values.

Sensitivity of individual tests was defined as the ratio of those with a positive test, divided by the total number of people with an active *Schistosoma* infection. As circulating antigen detection has been shown to be highly specific, active infection was defined as the demonstration of faecal egg excretion or the detection of CAA or CCA in either serum or urine.

Spearman's rank test was used for testing concordance, the Mann-Whitney test and the chi-square analysis to calculate differences between groups. Data were analyzed using SPSS/PC$^+$ statistical package on an IBM PC compatible computer.

3. Results

In total, 389 residents of Catharina Sophia were included in the study: 190 males and 199 females with a median age of 23 years (range 1–85). Schistosome specific IgM antibodies were tested in 295 individuals, 158 (54%) of which were found to be positive, with titres ranging from 16 to 2048 (median 128). Fig. 1a depicts the antibody titres at different age classes, showing a relatively low number of antibody positives in children under the age of 10 years old.

Table 1 summarizes the data on the diagnosis of an active *Schistosoma* infection, defined by the demonstration of faecal egg excretion or the detection of circulating antigens in either serum or urine. Parasitological examination was performed in 305 subjects. Live *S. mansoni* eggs could be demonstrated in 87 individuals (29%), with 56 cases showing an egg-output of less than 100 eggs per gram faeces (epg). Sixty-two percent of the egg excreters were males and both the percentage of positives and the intensity of infection were significantly higher in males than in females ($\chi^2 = 8.2$, $P < 0.01$; Mann-Whitney, $P < 0.001$, $n = 305$). Fig. 1b depicts the parasitological findings in different age classes. Both prevalence and intensity of infection rise with increase of age, showing a peak level in the age group of 40 to 49 and a decline thereafter.

Concerning the detection of circulating antigens, the highest percentages of positives were found for CCA in urine and CAA in serum, i.e. 28% and 23%, respectively (Table 1). In those cases positive, the levels of circulating antigen were generally very low. Fig. 1c and 1d represent serum CAA and urine CCA levels at different age classes. The pattern generally resembles that of the egg output, although with a less pronounced peak at the age around 40 years old. In males, serum CAA and CCA levels were significantly higher than in females, but for CCA in urine no differences were found between the two sexes.

In summary, 43% of the study population (169 cases) showed an active *S. mansoni* infection. A positive titre for at least one of the circulating antigens was found in 149 subjects of which 67 were at the same time positive for the parasitological examination. However, to enable a good comparison between the diagnostic performance of the individual assays, a selection had to be made based on the participation in all diagnostic tests.

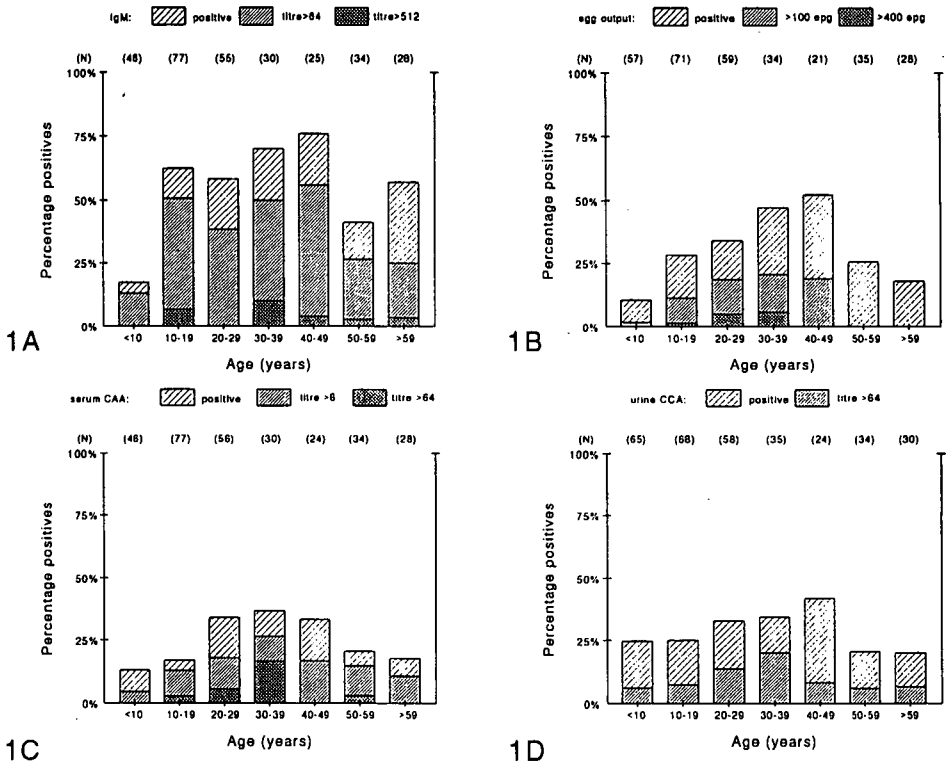

Fig. 1. Percentages of positives at different age classes of the population of Catharina Sophia, determined by the following diagnostical methods: **(A)** measurement of IgM levels by IFA, $n=295$; **(B)** counting of faecal egg excretion, $n=305$; **(C)** quantification of circulating anodic antigen (CAA) levels in serum, $n=295$, and **(D)** quantification of circulating cathodic antigen (CCA) levels in urine, $n=314$. Test results are arbitrarily divided into two or three categories.

TABLE I

Schistosoma mansoni egg output and circulating antigen levels at the population of Catharina Sophia, Surinam ($n=389$). Data are expressed as the number of individuals screened, the number and percentage of subjects found to be positive, and the minimum, median and maximum values of the positive cases

Screening method		*n*	Positives			Values		
			n	%	95%CI	min	med	max
stool[1]		305	87	29	23–34	10	70	550
CAA	serum[2]	295	69	23	19–28	4	16	4096
	urine	311	8	3	01–04	1.6	1.6	12.8
CCA	serum	291	49	17	13–21	8	8	32
	urine	314	87	28	23–33	32	64	512

[1] Duplicate Kato, values expressed as eggs per gram faeces

[2] Values expressed as titres

Abbreviations: n = number; 95%CI = 95% confidence interval; min = minimum; med = median; max = maximum; CAA = circulating anodic antigen; CCA = circulating cathodic antigen.

The 204 individuals selected for further analysis, showed the same egg-excretion and sex distribution as the non-selected cases (Mann-Whitney, $n=389$, $P>0.36$), but were slightly older in age (Mann-Whitney, $n=389$, $P=0.02$). An active *S. mansoni* infection could be demonstrated in 107 cases (52%) of which 46 were both positive for at least one of the circulating antigens as well as for the faecal examination, 49 had detectable antigen levels while no eggs were found and 12 cases excreted eggs while no circulating antigens could be demonstrated. Of the latter, all subjects excreted less than 100 epg. Only one patient was found to be positive for all four circulating antigen assays.

Table 2 summarizes the performance of each circulating antigen assay for the different egg output classes. For all immunodiagnostic assays the percentages of positives increased with increasing egg excretion.

The highest sensitivity was achieved with the urine-CCA assay, capable of identifying 59 of the 107 active cases (55%); immediately followed by the parasitological examination with 58 cases positive (54%) and the serum-CAA assay with 47 cases (44%). One hundred cases (93%) were positive for at least one of these three diagnostic tests and Table 3 shows their distribution. The additional 7 individuals all showed detectable CCA levels in serum, but were negative for CAA in urine. Fig. 2 compares for different age classes the prevalence rates obtained with parasitological examination and circulating antigen determination.

TABLE 2

Performance of four circulating antigen assays and specific antibody test in 204 individuals of Catharina Sophia who participated in all diagnostic assays. Data are expressed as the numbers and, between parentheses, the percentages of positives

Egg output	*n*	s-CAA	u-CAA	s-CCA	u-CCA	IgM
Negative	146	20 (14)	1 (0.7)	10 (7)	23 (16)	61 (42)
≤ 100 epg	39	13 (33)	4 (10)	8 (21)	23 (59)	29 (74)
> 100 epg	19	14 (74)	2 (11)	13 (68)	13 (68)	15 (79)
Total	204	47 (23)	7 (3)	31 (15)	59 (29)	105 (51)

Abbreviations: *n* = number; epg = eggs per gram faeces; s = serum; u = urine; CAA = circulating anodic antigen; CCA = circulating cathodic antigen.

TABLE 3

Frequency of diagnostic tests among 100 cases with positive test results for demonstration of *S. mansoni* eggs in faeces, circulating anodic antigen (CCA) in serum and circulating cathodic antigen (CCA) in urine

Test	Number positive
eggs only	15
CAA serum only	19
CCA urine only	22
CAA serum/CCA urine	1
eggs/CAA serum	7
eggs/CCA urine	16
eggs/CAA serum/CCA urine	20
Total	100

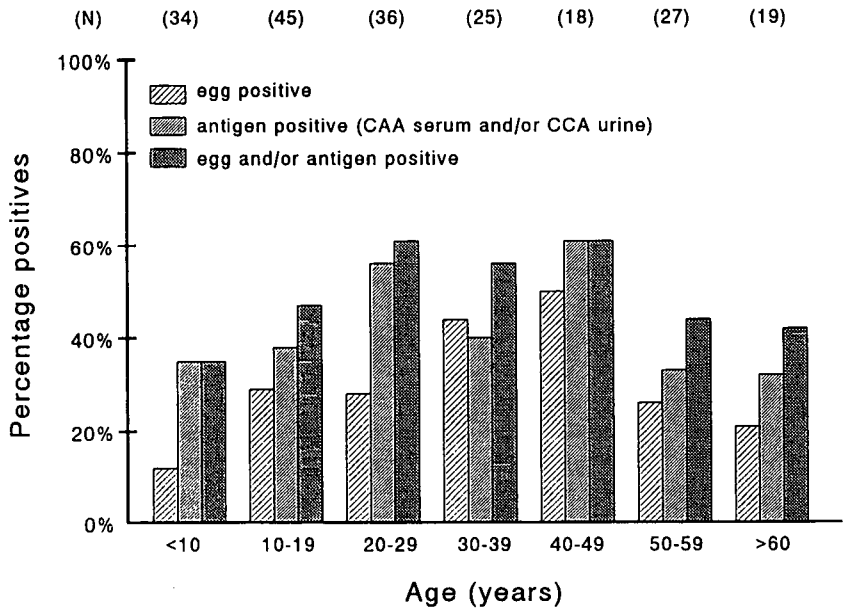

Fig. 2. Comparison of schistosomiasis prevalence rates at different age classes obtained with either parasitological examination or circulating antigen detection in 204 individuals of Catharina Sophia who participated in all diagnostic assays.

Table 2 also shows that specific IgM levels could not be demonstrated in all individuals with detectable egg excretion. Out of the 107 cases with a proven active infection, 40 had no detectable antibodies (37%). These IgM negatives showed significantly lower egg excretion compared to the IgM positives (Mann-Whitney, $n=107$, $P<0.01$), but no differences were found in age or in serum CAA or urine CCA levels (Mann-Whitney, $n=107$, $P>0.32$). In 38 cases, specific IgM antibodies were found while no active *S. mansoni* infection could be demonstrated. No differences were found in age or IgM titre between those with or without proven active infection.

Significant correlations were found between the number of *S. mansoni* eggs in faeces and the levels of circulating antigens, except for CAA in urine (Spearman's $\rho>0.42$, $P<0.001$, $n=204$). The highest correlation coefficient was found with serum CCA ($\rho=0.54$) and the lowest with serum CAA ($\rho=0.43$). Except for urine CAA, the level of circulating antigens were also related with each other (Spearman's $\rho>0.26$, $P<0.001$, $n=204$).

4. Discussion

For epidemiological studies, active *Schistosoma* infections are usually diagnosed by a stool analysis, demonstrating parasitic eggs in faeces, often by using a Kato examination. Because of its low sensitivity, this method should be repeated several times when applied in areas with low transmission levels, to give an adequate

indication of the true prevalence (Polderman et al., 1985; Gryseels et al., 1991; De Vlas and Gryseels, 1992).

In this study we applied circulating antigen detection during a survey in one single village in the district of Saramacca. This area used to have a low transmission of *S. mansoni*, although little is known about current infection levels. From Fig. 1 and Table 1 and 2, it is clear that the transmission in Catharina Sophia is still low, with light infections in the majority of positive cases. Parasitological examination revealed a prevalence of 29%, which is in concordance with previous findings (Van der Kuyp, 1969; Deelder et al., 1980a). The higher prevalence in males compared to females and in adults compared to children, has also been reported before (Van der Kuyp, 1969).

Specific antibody detection showed positive IgM levels in 54% of the study population, primarily with titres considerably higher than the cut-off level. This suggests that substantially more individuals are, or have been, exposed to schistosome infection than suggested by parasitological examination only. However, when these data were analyzed in more detail, some discrepancies were found. Positive IgM levels were determined in as many as 38 out of the 97 cases without any sign of an active infection. This indicates either a past infection or a very low intensity, missed by the other diagnostical techniques. Much more difficult to interpret is the fact that 24% of all egg excreters had no positive IgM levels; of all cases showing an active infection this was even 37%. It can not be ruled out that these subjects have specific antibodies of other isotypes, although previous studies have shown that the IFA technique as used here is not only highly specific, but also very sensitive. Indeed, continuously high IgM titres during follow-up periods of several years have been reported (Deelder et al., 1989c). False-negative antibody levels were also found in a previous study performed in Surinam, where 11% of the individuals with egg excretion showed negative IgM titres in the IFA. These cases were mainly adults (Deelder and Kornelis, 1980). In our study, false negative IgM titres did not occur significantly more in adults than in children. In another study, several immunodiagnostic assays for the detection of IgG (H+L) antibodies were compared in an Ethiopian population with a *S. mansoni* prevalence of 65%. False-negative reactions occurred in 17% of the cases and generally antibody titres decreased with an increase of age. Data were explained by regional differences, since these results could not be confirmed by using the same techniques on serum samples from other areas (Polderman and Deelder, 1977).

With circulating antigen determination, the prevalence of an active infection was 43%. In 49 individuals, positive circulating antigen levels were found while no faecal egg excretion could be demonstrated. The insufficient sensitivity of the Kato examination is illustrated by the fact that eggs could be found in 9 out of 30 negative subjects when applying a more sensitive stool examination procedure (Polderman et al., 1994). The "true" prevalence of 43% we found in this study is completely in concordance with the prevalence found in the same study population after more sensitive stool examination, as well as with the theoretical prevalence, which could be predicted by applying the pocket-chart, recently developed by De Vlas and colleagues (Polderman et al., De Vlas et al., 1993).

Of the circulating antigen assays, the highest sensitivity was achieved with the urine-CCA assay (55%), followed by the serum-CAA assay (44%), while 79% of the active cases were positive for at least one of these two tests. Improvement of sensitivity by parallel testing on more than one antigen has been reported before in

a study on Egyptian schistosomiasis patients (Van Lieshout et al., 1992). Also in that study, cases excreting less than 100 *S. mansoni* epg were analyzed separately. The sensitivity of the four individual circulating antigen assays was much higher than at the present study, ranging from 75 to 85%, while 95% showed positive titres for serum CCA in combination with one of the other assays. This is in line with our previous observation that the sensitivity of the assays is not only depending on the intensity of infection, but also on the prevalence in the area where the patients come from (De Jonge et al., 1988). On the other hand, parasite strain differences in the production of CAA and CCA can not be ruled out. One previous study on schistosomiasis mansoni patients from South America revealed 66% of the serum samples to be positive for CAA and 83% for CCA, but the infection levels of these patients were high (De Jonge et al., 1991).

Recently, circulating antigen detection was also applied in a newly established, heavily *S. mansoni* infected community in the northern part of Senegal, where faecal egg excretion was found in 91% of the population. Similar to this study, the urine-CCA assay was found to have the highest sensitivity, immediately followed by the serum-CAA assay, while CAA could only be demonstrated in 55% of the urine samples and in remarkably low concentrations. Results were explained by a possible difference in clearance dynamics of this antigen due to the very recent nature of the infection in this specific study population (Polman et al., 1995). The fact that in our study, with long standing infections and low egg excretion, levels of CAA in urine were also found to be very low, indicates that possibly other mechanisms may play a role in this phenomenon.

Consistent with previous studies, we found a significant correlation between egg excretion and circulating antigen levels, except for CAA in urine. However, as the intensities of the infections were generally low, most of the positive antigen levels were very close to the cut-off level, which is a disadvantage for the clinical or epidemiological application of the assays. Therefore further development of the assays will require improvement of the detection limit.

Based on the results of this study, we conclude that for the diagnosis of schistosomiasis in low endemic areas, circulating antigen detection should be considered as a complementary tool rather than as a substitute for parasitological examination. Specially the detection of CCA in urine and CAA in serum supplies additional information to determine more accurately the "true" prevalence of infection.

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Chapter 2.7

Detection of the circulating antigens CAA and CCA in serum and urine in a group of Dutch travellers with acute schistosomiasis.

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Detection of the circulating antigens CAA and CCA in a group of Dutch travellers with acute schistosomiasis.

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Abstract

Detection of circulating anodic antigen (CAA) and circulating cathodic antigen (CCA) in serum and urine has shown to be a highly specific and sensitive alternative for the diagnosis of schistosome infections in endemic areas. However, it is not known how soon after the onset of infection these antigens can be demonstrated in humans. Also, not much research has been done on the detection of these antigens in individuals normally living in non-endemic areas. Here we studied the kinetics of CAA and CCA in serum and urine of a group of 28 Dutch tourists, shortly after accidental exposure to a *Schistosoma* infection during a visit to Mali. Twenty-seven of them were found to be positive for *Schistosoma* eggs and/or specific antibodies. From each individual, one to four serum samples were tested on circulating antigen level, 4 to 15 weeks after exposure, and from 22 subjects also a urine sample was tested. CAA and CCA levels were quantified by monoclonal antibody based ELISA's and TRIFMA's. In serum, 23 individuals (85%) were positive at least once for one or both antigens, but titres were generally very low. CAA and CCA could be demonstrated starting from 5 or 6 weeks after exposure, respectively. Urines were all found to be negative. Follow-up after chemotherapy showed almost all cases to be negative after 7 months.

Introduction

Quantification of the adult worm antigens CAA (circulating anodic antigen) and CCA (circulating cathodic antigen) in serum and urine has shown to be a valuable alternative for the diagnosis of active *Schistosoma* infections in endemic areas.[1-4] Antigen levels are related to intensity of infection, as expressed by parasite egg excretion. In addition, studies with hospitalized patients demonstrated CAA and CCA levels to drop to zero within a period of 3 to 6 weeks following successful chemotherapy.[5-7]

To distinguish between failure of treatment and reinfection in those individuals who are continuously exposed, it is important to know how soon after the onset of infection antigens can be detected. Animal studies showed that CAA and CCA can be demonstrated in serum 2½-5 weeks after exposure, also depending on the intensity of infection.[8-10] However, in experimental models, worm loads are high compared to the human situation, and data can not simply be extrapolated.[11] One human case study showed CAA to be demonstrable in serum 4 weeks after exposure.[12]

In endemic areas, it is almost impossible to determine the exact date of the onset of infection, as exposures are usually not limited to a single event. In this study, we screened a group of Dutch travellers who got accidentally exposed to *Schistosoma* infection in a single event during a visit to Mali.[13] Following this incident, serum and urine samples could be collected starting from weeks 4 and 8, respectively. All infected subjects were treated 3 months after exposure and then followed during 14 months. Serum and urine samples were tested for CAA and CCA levels, not only to define the first moment from which these antigens can be demonstrated, but also to study the diagnostic application of CAA and CCA determination in individuals normally living in non-endemic areas. Results were compared with previously described findings on parasitological, clinical and serological examination.[13,14]

Materials and methods

Study population

We studied 28 out of a group of 29 travellers, who got accidentally exposed to a *Schistosoma* infection by swimming in freshwater pools in the Dogon area of Mali, West Africa.[13] One subject was left out because he had been exposed during another day and at another location. The study population consisted of 9 males and 19 females, ranging in age between 26 and 58 years (median 42).

Intensive parasitological examination (including sedimentation techniques)[15] and serological screening (detection of specific antibodies) revealed all 28 exposed subjects except one to be infected. Schistosome eggs were found in stool and/or urine of 22 individuals: those of *S. mansoni* in 19 cases, *S. haematobium* in 7 and terminally spined eggs in 10 cases.[13] Anti-gut associated IgM antibodies were demonstrated in serum of all 27 infected travellers, while anti-SEA (soluble egg antigen) antibodies were found in 24 of them.[14] Fifteen individuals had symptoms and signs characteristic for Katayama syndrome.[13]

Sample collection

One to four serum samples were collected from each subject, 4 to 15 weeks after the date of exposure. From 22 individuals, also a single urine sample was collected at week 8 to 16 (median 11). All infected individuals were treated 12 to 16 weeks after exposure with praziquantel (40 mg/kgbw) and re-examined approximately 7 weeks, 7 months and 14 months after chemotherapy.[13]

For comparison, we also tested 135 serum samples and 80 urine samples from Dutch individuals who had no history of visiting an area where schistosomiasis is endemic.

Circulating antigen determination

Serum and urine samples were tested on CAA and CCA levels by sensitive and highly specific monoclonal antibody based sandwich assays as described by Deelder *et al.* and De Jonge *et al.*.[16,17] For reference, serial dilutions of the trichloroacetic acid-soluble fraction of adult worm antigen (AWA-TCA) were assayed simultaneously on each plate. AWA-TCA contains approximately 3 % (w/w) of CAA and 3 % of CCA, as determined by using immunopurified antigen preparations [18,19].

Before testing, samples were pretreated to dissociate immune complexes and/or to remove interfering proteins. Serum samples were pretreated with trichloroacetic acid (TCA),[20] and thereafter tested in an ELISA system in doubling dilutions, starting from 1/4.[16,17] Antigen levels were expressed as titres, viz. the reciprocal value of the highest sample dilution showing a staining above the background (mean absorbance of 12 buffer controls plus three times the standard deviation). Sera showing for CAA a titre >2 were considered to be positive, as all tested negative controls showed a titre ≤ 2.[21] As 98.5% of the negative serum samples showed for CCA a titre ≤ 4, samples were considered to be positive for CCA with a titre >4.[21]

Case	weeks after exposure											
	4	5	6	7	8	9	10	11	12	13	14	15
1	—	—		—					—/8			
2	—			8/8								
3		—		4/—								
4		—							—/16			
5		—							—/16			
6		8/—	—									
7			—					—	—			
8			—									—
9			—							—/32		
10			—									
11			—/32									
12			4/16									
13				16/—								
14				4/8					4/32			
15				32/16					8/—			
16					4/—							
17					8/—				8/—			
18					8/16					—		
19					4/8					—		
20						—						
21						—		32/—				
22						—						
23						—/16						
24								64/—				
25								8/—				
26									—/16			
27										16/8		
28											4/—	

Figure 1. Serum levels of circulating anodic antigen (CAA) and circulating cathodic antigen (CCA) in 28 Dutch tourists after single exposure to *Schistosoma* infection. Antigen levels are expressed as titres of CAA/CCA, '—' indicates no antigen could be demonstrated.

In an attempt to increase the number of demonstrated positive cases, an alternative pretreatment procedure was applied for the urine samples. Recently, improved sensitivity of the CAA urine assay was shown by a heat-incubation step in alkaline buffer.[21] In addition, urine levels of CAA and CCA were determined by time-resolved immunofluorometric assays (TR-IFMA's), which are in general similar to the CAA and CCA ELISA, but use europium labelled streptavidin in the conjugate step.[22] TR-IFMA's show slightly better detection limits compared to the CAA and CCA ELISA's.[23] For serum, not enough material was available to retest all the samples. Urines were tested in the TR-IFMA in duplicate, at a final dilution of eight times. Antigen levels were expressed as concentrations, calculated with the 4-parameter curve fitting method. As 98.5% of the control urines showed a concentration lower than 28 ng AWA-TCA/ml and 32 ng AWA-TCA/ml for CAA and CCA, respectively, urines were considered to be positive when showing a concentration higher than this cut-off level.

Results

Figure 1 depicts the individual serum CAA and CCA titres, 4 to 15 weeks after exposure. One subject (number 8) who was negative for parasite eggs and specific antibodies,[13,14] was also found to be negative for CAA and CCA. Sixteen of the 27 infected cases (59%) became positive for CAA in serum at least once, starting 5 weeks after exposure, with titres ranging from 4 to 64 (median 8). For CCA, 14 subjects (52%) were positive at least once, starting from 6 weeks after exposure, with titres ranging from 8 to 32 (median 16). In total 23 (85%) individuals became positive for at least one of the two antigens. Of the four remaining negative cases, 3 showed *Schistosoma* eggs after intensive parasitological examination.[13] No correlations were found between circulating antigen levels and parasitological findings or between circulating antigen levels and the presence of Katayama syndrome. All urines were found to be negative for CAA and CCA (data not shown).

The 23 cases positive for serum CAA or CCA, were followed for up to 14 months after treatment with praziquantel. Intensive parasitological examination

revealed that 6 of them were not completely cured,[13] 4 of them were also positive for CAA and/or CCA 7 weeks after treatment (Figure 2). Three cases were retreated and tested again: they all became negative for CAA or CCA.

Nine additional cases showed low but positive CAA or CCA titres 7 weeks after chemotherapy, while no parasite eggs were demonstrated. All individuals, except two, were found to be negative at 7 and 14 months (Figure 2).

	follow-up after treatment				
Case	A		B		C
[1]1	—	RT	—		—
2	—		—		
3	—		—		—
[1]4	4/16		—	RT	—
[1]5	—/16		—		
6	8/8		—		
[1]9	16/16				
11	—/8		—		
[1]12	—/8		—		
13	—		8/—		8/—
14	—/16		—		
15	—		—		—
16	—/8		—		
17			—		—
18	4/—		—		4/—
19	4/—		—		—
21	—/8		—		—
23			—		—
24	—/8		—		
[1]25	—	RT	—		—
26	—				—
27			—		
28	—/8		—		—

Figure 2. Follow-up of serum levels of circulating anodic antigen (CAA) and circulating cathodic antigen (CCA) in a group of 23 Dutch tourists after treatment with praziquantel. Antigen levels are expressed as titres of CAA/CCA, '—' indicates no antigen could be demonstrated. Abbreviations: A = 5-15 weeks, median = 7 weeks; B = 20-42 weeks, median 29 weeks (7 months); C = 51-62 weeks, median 61 weeks (14 months); RT = retreated with praziquantel; [1] Parasitologically positive during the follow-up after treatment.

Discussion

For the parasitological diagnosis of light *Schistosoma* infections, intense and repeated screening is often needed, as the number of excreted eggs is relatively small. An alternative method is serological diagnosis by the detection of specific antibodies, which has shown to be a very sensitive and specific technique.[24,25] However, antibody levels are generally not related to the intensity of infection and they cannot be used to differentiate current from past infections.[26-28] Consequently, antibody detection is not the appropriate technique for the assessment of cure or for the diagnosis of reinfection. This while there is an expanding need in industrialized countries for relatively simple and rapid diagnostic procedures which can demonstrate an active *Schistosoma* infection. Due to an increasing popularity to travel to the tropics, the number of individuals originating from non-endemic areas who get exposed to a *Schistosoma* infection is growing.

For this purpose, a promising alternative would appear the determination of schistosome circulating antigens, such as CAA and CCA.[4] Based on studies on populations living in endemic areas, the levels of these antigens were found to be related to the presence of an active infection and following successful treatment, CAA and CCA levels were found to become negative within 3 to 6 weeks.[5,29]

Serum CAA levels in individuals originating from non-endemic countries have been studied once before in a group of 82 Dutch schistosomiasis patients who all showed specific antibodies.[28] The composition of this group was very heterogeneous, the date and place of exposure were unknown in a majority of the cases. Low, but demonstrable CAA levels were only found in 21% of the study population. In addition, one post-chemotherapy sample was examined for each case, but the period between chemotherapy and post-treatment examination ranged from 88 to 612 days. All individuals became negative for CAA after chemotherapy, while the levels of specific antibodies stayed high.[28]

Intensities of infection were also very low in the present study. For example, of the 19 cases showing *S. mansoni* eggs, 10 individuals showed less than 10 eggs per gram of faeces (epg), and 5 between 10 and 100 epg (Polderman *et al.*, unpublished data). Although 85% of the cases were at least once positive for circulating antigen in serum, CAA and CCA levels were generally close to the cut-off values of the assays involved. Probably due to small variations in antigen levels, a few positive cases became negative again when tested a second time. This also relates the chance to diagnose a person as positive to the number of samples screened.

When re-examined approximately 7 weeks after chemotherapy, 13 out of

the 23 cases were still positive for CAA and/or CCA. Based on previous post-treatment studies on circulating antigens, this would indicate a high failure of treatment. However, after exceptionally intensive parasitological examination, eggs could be demonstrated in only 4 subjects, and all but two cases were negative for CAA and CCA when tested again at 7 and 14 months. This suggests a difference in CAA and CCA kinetics following chemotherapy in these lightly infected individuals, compared to cases living in endemic areas.

Our finding that CAA and CCA could be demonstrated in serum 5 to 6 weeks after exposure, corresponds with experimental data and with a single human case study.[8,9,12] As our study population harboured light intensities of infection, this time period may even be shorter in individuals living in endemic areas, who generally have higher worm loads. This corresponds with the number of weeks needed for the assessment of cure and may therefore hamper the use of circulating antigen determination to distinguish failure of treatment from reinfection in areas with an ongoing transmission.

In conclusion, while both CAA and CCA could be demonstrated in humans several weeks after exposure, determination of these antigens seems to be, for the moment, of no importance for routine diagnosis of schistosomiasis in travellers returning from endemic areas with a low parasite worm burden. The sensitivity of CAA and CCA determination was found to be similar to intensive parasitological screening, but lower than the detection of specific antibodies. Analogous to parasitological examination, antigen levels were only slightly above detection limits, which may also hamper the practical application of CAA and CCA determination in populations as described here. Although it might be possible that no detectable CAA and CCA is present at all in the circulation of individuals with acute schistosomiasis, recent studies (Deelder *et al.*, unpublished data) suggest that a further optimization of the assay not only results in an improvement of the detection limits, but also in a slight enhancement of the diagnostic performance.

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Chapter 2.8

A simple technique to pretreat urine and serum samples for quantitation of schistosome circulating anodic and cathodic antigen.

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A simple technique to pretreat urine and serum samples for quantitation of schistosome circulating anodic and cathodic antigen

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For the detection of the circulating schistosome antigens CAA (circulating anodic antigen) and CCA (circulating cathodic antigen) in serum and urine samples of *Schistosoma* infected individuals, pretreatment of samples with trichloroacetic acid (TCA) is a standard procedure. In the present study several methods were evaluated in order to develop a more simple and rapid technique than the – especially for pretreatment of urine samples – laborious TCA technique. Optimal results were obtained with a method in which serum or urine samples were pretreated by a heat-incubation step (70°C, 30 min) in an alkaline buffer (pH 9.6). In a comparison of the new technique with the TCA pretreatment, serum and urine samples of *S. mansoni* infected individuals from Zaire ($n = 80$) and of uninfected controls from The Netherlands ($n = 208$) were pretreated and assayed for CAA and CCA. Both pretreatment techniques showed similar sensitivities and specificities for CAA and CCA in serum, and CCA in urine. However, for the determination of CAA in urine the new technique performed significantly better, resulting in an increase of the sensitivity from 32 to 70% (titre determination).

Key words: Schistosomiasis; Urine; Serum; Pretreatment; ELISA; Circulating anodic antigen; Circulating cathodic antigen

Introduction

Quantitative immunodiagnosis of human schistosome infections by circulating antigen detection is increasingly recognized as a feasible and valuable approach (Deelder, 1984; Hayunga et al., 1986; Feldmeier et al., 1986; De Jonge et al., 1988, 1990; Deelder et al., 1989). Most studies have been concentrated on the demonstration of circulating anodic antigen (CAA) and circulating cathodic antigen (CCA), both adult worm gut-associated glycoconjugates. CAA and CCA are genus-specific antigens and have been demonstrated in serum and urine of individuals infected with *Schistosoma mansoni*, *S. haematobium*, *S. intercalatum* and *S. japonicum* (De Jonge et al., 1989b, 1989c, 1991a; Van 't Wout et al., 1992; Van Lieshout et al., 1992). The levels of these antigens were shown to be related to the intensity of infection and a rapid clearance after successful chemotherapy has been demonstrated (De Jonge et al., 1989a, 1991b; Van Lieshout et al., 1991).

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However, before CAA and CCA can be reproducibly quantified in a sensitive monoclonal antibody-based sandwich ELISA, serum and urine samples need to be pretreated with trichloroacetic acid (TCA) to dissociate immune complexes for serum and to remove interfering components (De Jonge et al., 1987).

For the determinations of CAA in urine, samples even need to be concentrated, a time-consuming and expensive step, necessary because of the low CAA level in this type of specimen. For the CCA assay this concentration step is not required, as CCA levels in urine are, in comparison to CAA, relatively high and titres up to 8 may be found in urine samples from individuals from non-endemic areas, necessitating a cut-off titre of 8 (Van Lieshout et al., 1992).

The aim of the present study was to compare several alternative pretreatment methods for both serum and urine, which could lead to a higher throughput, use of a smaller sample size and a simplification of the whole assay.

After adopting one technique, we examined the levels of CAA and CCA, expressed as titres and concentrations, in a group of individuals from Tshamaka, an area endemic for *S. mansoni* in Zaire, and in a group of Dutch uninfected individuals.

It was found that one simple pretreatment technique could be adopted for both serum and urine, giving essentially the same results as with TCA pretreatment for CAA and CCA serum, and CCA urines, and even significantly better results for CAA detection in urine samples.

Materials and methods

Study population

To determine the sensitivity and specificity of the different pretreatment procedures, serum and urine CAA and CCA levels were compared in samples from two different groups of individuals.

The first group consisted of 49 serum samples and 50 urine samples from 80 inhabitants of Tshamaka, a village in Maniema, Eastern Zaire. The endemic situation for schistosomiasis mansoni in this area has been described in detail by Polderman et al. (1985a; 1985b). The prevalence of *S. mansoni* in this region is 95% and the faecal egg excretion of the group studied here ranged from 7 to 15 440 eggs per gram faeces (epg) (median 410 epg, 90th percentile 3981).

The second group consisted of 106 serum samples and 102 urine samples from 208 healthy Dutch individuals, who had no history of visiting an area where schistosomiasis is endemic.

Buffers and chemicals

Pronase from *Streptomyces griseus* and bovine serum albumin (BSA) fraction V were purchased from Boehringer Mannheim, Mannheim, Germany. Polyethylene glycol-1000 (PEG-1000) and Tween-20 were obtained from Merck, Schuchardt, F.R.G.. All other chemicals were of analytical grade.

Assay buffer was 0.035 M phosphate buffered saline (PBS) pH 7.8 containing 0.5% (v/v) PEG-1000 and 0.02% (v/v) Tween-20. Pronase solution was 0.1% (w/v)

pronase dissolved in 0.035 M PBS (pH 7.8). Acidic buffer was a 0.5 M glycine-HCl buffer (pH 2.4). Alkaline buffer was a 0.244 M sodium carbonate buffer (pH 9.6).

Pretreatment methods

Five different pretreatment methods for serum and urine, all incorporating a heating step of 30 min at 70°C, were compared with the previously published TCA pretreatment method (De Jonge et al., 1987).

In the first method, 200 µl undiluted urine, undiluted serum or 10-fold diluted serum (20 µl sample + 180 µl assay buffer) were heated.

In the second method, 100 µl urine + 100 µl pronase solution or 20 µl serum + 20 µl pronase solution in 160 µl assay buffer were incubated for 1 h at 37°C and then heated.

In the third method, 50 µl urine or 20 µl serum were mixed with an equal volume acidic buffer, heated and then neutralized with 100 µl or 160 µl alkaline buffer, respectively.

In the fourth method, based on a method described by Dhar and Ali (1992) for the detection of testosterone in serum, 100 µl urine or 50 µl serum were mixed with 100 µl or 150 µl alkaline buffer, respectively, and heated.

In the fifth method, 100 µl urine or serum sample were mixed with 50 µl 1.0 M NaOH, heated and neutralized with 50 µl 1.0 M HCl.

After all five pretreatment methods, samples were mixed well and used directly in the ELISA. The TCA-treated urine samples were dialysed, lyophilised and reconstituted in assay buffer before use.

A panel of urine and serum samples from six *S. mansoni* infected and from six uninfected individuals was tested for CAA and CCA levels for all different pretreatment procedures.

Determination of circulating antigens

Antigen levels were expressed in titres as well as in concentrations of CAA or CCA (pg antigen/ml), calculated from a standard dilution curve of the lyophilized TCA-soluble fraction of the adult worm antigen (AWA-TCA), a preparation primarily containing carbohydrates. CAA and CCA each constitute approximately 3% (w/w) of AWA-TCA (Van Dam, personal communication).

CAA was assayed by a monoclonal antibody based enzyme-linked immunosorbent assay as described by Deelder et al. (1989). The lower detection limit of this ELISA is 48 pg CAA per ml, and the linear range 94 to 750 pg CAA per ml. CCA was measured using the ELISA described by De Jonge et al. (1990), which has a lower detection limit of 120 pg CCA per ml, with a linear range of 230 to 1875 pg CCA per ml. Both ELISAs were performed in flat bottom 96-well polystyrene microtitration plates, Maxisorb (Nunc, Denmark) and plates were read on an automatic microtitre plate reader (EL 311, Biotek Instruments, Winooski, USA).

Urine and serum samples were tested in doubling dilutions. The serum samples were tested from a 1/4 dilution onwards. Urine samples were tested from a 1/2 dilution, except for the TCA-treated urine samples tested for CAA, which were used from a 5-fold concentration. For both ELISAs, the reciprocal value of the highest

sample dilution showing a colouring above the background (mean absorbance of 12 buffer controls plus 3-times the standard deviation) was taken as the end-point titre. Concentrations were calculated with the four-parameter curve fitting method. Software package used was KinetiCalc V 2.03 (Biotek Instruments, USA).

Data analysis

Because none of the data were continuously or normally distributed, non-parametric methods were used to describe and evaluate the results. Data were characterized by their minimum and maximum values, medians and 90-percentile values; Spearman's rank correlation was used for the calculation of the concordance between egg output and titres or concentrations. The statistical package SPSS/PC+ (SPSS, Chicago, IL, USA) was used on an IBM-PC/AT compatible computer.

In the present study, for each assay the cut-off level was chosen such that a specificity of at least 98% was achieved. Specificity is defined as the percentage of correctly identified negatives; the term sensitivity is used to define the percentage of assay test-positives among the total number of infection-positive individuals.

Results

To compare the five different pretreatment techniques with the TCA pretreatment method, CAA and CCA levels were determined in a panel of urine and serum samples from six *S. mansoni* infected and six uninfected individuals, after pretreatment with the respective techniques.

Simply heating the samples for 30 min at 70°C did not work well with the undiluted serum samples because of coagulation of the proteins. Ten-times diluted serum samples gave false positive results of the uninfected individuals. However, the CAA levels of urine samples treated in this way correlated well with egg output and results obtained with the TCA pretreatment. Pretreatment with pronase or acidic buffer resulted in false positive results with the urine samples of the uninfected individuals. On the other hand treatment of serum samples from infected individuals with pronase or sodium hydroxide resulted in CAA and CCA levels which showed no correlation at all with those found after the TCA pretreatment (data not shown).

Serum and urine samples heated in alkaline buffer generated results which correlated both with egg output and with the results obtained with TCA pretreatment. Therefore, this technique was adopted to pretreat all serum and urine samples and to compare CAA and CCA levels, thus obtained, with the TCA-pretreatment method (Table 1 and 2).

For serum samples, at a specificity of at least 98%, cut-off levels for samples from uninfected controls were nearly equal for both pretreatment techniques. Likewise, with samples from infected individuals similar sensitivities were found (Tables 1 and 2).

For urine samples, at a specificity of at least 98%, the cut-off level for CCA determinations was at least 4-times lower with alkaline pretreatment than with TCA pretreatment, with similar sensitivities. For CAA determinations in urine, at a 98% specificity level, similar cut-off levels were found, but the alkaline pretreatment

TABLE 1

Detection of CAA and CCA in urine ($n = 50$) and serum ($n = 49$) samples pretreated in alkaline buffer or with TCA: distribution of titres, cut-off values, specificity and sensitivity

Abbreviations: min = minimum; max = maximum; med = median; 90-p = 90th percentile value; spec = specificity; sens = sensitivity; CAA = circulating anodic antigen; CCA = circulating cathodic antigen; alk = alkaline pretreatment; TCA = trichloroacetic acid pretreatment. All values are expressed as titres, except for the specificity and sensitivity, which are expressed as percentages.

Sample	Treatment	Titres					Spec. (%)	Sens. (%)
		min	max	med	90-p	cut-off		
CAA								
Serum	alk	<4	8192	128	2048	<4	98.0	87.8
	TCA	<4	2048	64	486	<4	100.0	87.8
Urine	alk	<2	2048	8	128	<2	99.0	70.0
	TCA	<0.2	51.2	0.4	12.2	0.8	98.6	32.0
CCA								
Serum	alk	<4	1024	32	128	4	98.0	75.5
	TCA	<4	2048	32	512	4	98.0	69.4
Urine	alk	<2	8192	128	2048	<2	98.0	96.0
	TCA	16	8192	256	3891	8	98.8	100.0

TABLE 2

Detection of CAA and CCA in urine ($n = 50$) and serum ($n = 49$) samples pretreated in alkaline buffer or with TCA: distribution of concentrations, cut-off values, specificity and sensitivity

Abbreviations: min = minimum; max = maximum; med = median; 90-p = 90th percentile value; spec = specificity; sens = sensitivity; CAA = circulating anodic antigen; CCA = circulating cathodic antigen; alk = alkaline pretreatment; TCA = trichloroacetic acid pretreatment. All values are expressed as ng antigen per ml, except for the specificity and sensitivity, which are expressed as percentages.

Sample	Treatment	Concentration (ng/ml)					Spec. (%)	Sens. (%)
		min	max	med	90-p	Cut-off[a]		
CAA								
Serum	alk	0.0	256.0	7.92	68.2	*	98.0	87.8
	TCA	0.0	110.2	7.23	45.0	*	100.0	87.8
Urine	alk	0.0	32.7	0.17	3.5	*	99.0	62.0
	TCA	0.0	1.6	0.04	0.6	0.05	98.9	38.0
CCA								
Serum	alk	0.0	122.0	5.91	40.9	1.52	98.0	79.4
	TCA	0.0	86.8	5.52	47.1	1.14	98.0	69.4
Urine	alk	0.0	4112.0	47.16	819.5	*	98.0	96.0
	TCA	5.3	1921.3	72.41	746.5	2.46	98.8	100.0

[a] * = lower detection limit.

resulted in a 70% sensitivity vs. 32% for TCA pretreatment (titre determination) (Tables 1 and 2).

Titre and concentration ranges for the serum and urine samples tested for CCA were equal for both methods. For CAA, urine samples treated with alkaline buffer

gave antigen levels which were at least 5-times higher than with TCA pretreatment (Tables 1 and 2).

For both methods Spearman rank correlations for CAA and CCA levels correlated well with the egg output (rho ranging between 0.51 and 0.84, $P<0.001$) (Table 3). As an example the correlation between egg output and CCA levels in urine samples treated with the alkaline buffer is shown in Fig. 1.

Discussion

The circulating antigens CAA and CCA have been demonstrated in serum and urine samples of *Schistosoma*-infected individuals from various endemic areas (De Jonge et al., 1988, 1989b, 1991a; Van Lieshout et al., 1992; Barsoum et al., 1991) and antigen detection assays are now recognized as having a strong potential for quantitative diagnosis of human schistosomiasis, and follow-up of chemotherapy (De Jonge et al., 1989b, 1991b; Van Lieshout et al., 1991).

A major drawback of this approach is the necessity of a – especially for urine samples – time-consuming TCA pretreatment procedure, which has stimulated us to develop a more simple technique. For the present study, individuals from the Tshamaka village from an endemic area with high infection intensity, were deliberately chosen because the egg output ranged from very low to very high (7 to 15440 eggs per g faeces) (Polderman et al., 1985a, 1985b).

On the basis of a pilot study, it was shown that for urine samples a pretreatment by heating alone (Spearman rank correlation between CAA level and epg = 0.71 $P<0.001$ $n=50$, data not shown) as well as a pretreatment in alkaline buffer could be used. For the pretreatment of serum samples only the pretreatment in alkaline buffer was possible. Because of the advantage of having one single technique for pretreatment of urine and serum samples, we adopted for all samples a heat treatment in alkaline buffer, as previously described for testosterone by Dhar and Ali (1992).

For serum samples this pretreatment gave the same results as the TCA pretreatment originally described by De Jonge et al. (1987) and now commonly adopted for

TABLE 3

Spearman rank coefficients of correlation between egg output and levels of CAA or CCA (titres and concentrations) determined after pretreatment with alkaline buffer or TCA.

(For all correlations $P<0.001$)
Abbreviations: CAA = circulating anodic antigen; CCA = circulating cathodic antigen; TCA = trichloroacetic acid pretreatment; alkaline = alkaline pretreatment; n = number of pair-wise observations.

	CAA				CCA			
	serum	n	urine	n	serum	n	urine	n
Titres								
TCA	0.79	49	0.55	50	0.76	49	0.70	50
Alkaline	0.81	49	0.72	50	0.78	49	0.72	50
Concentrations								
TCA	0.79	49	0.51	50	0.80	49	0.79	50
Alkaline	0.84	49	0.71	50	0.78	49	0.75	50

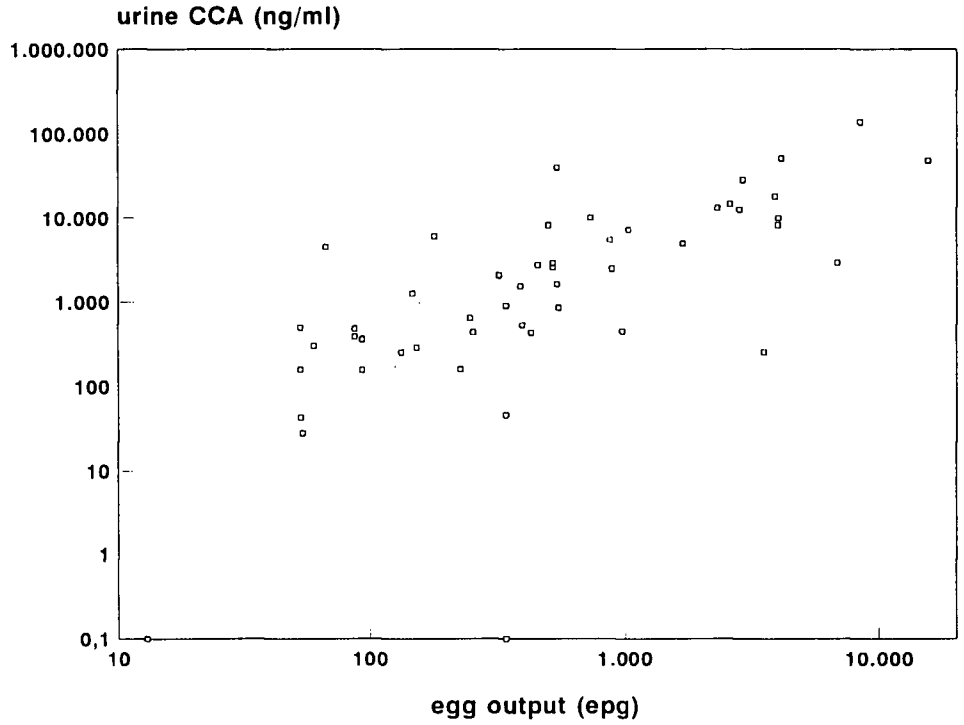

Fig. 1. Correlation of CCA concentration in urine samples, pretreated with alkaline buffer, with the egg output in *S. mansoni* infected individuals from Zaire (rho 0.75, $P < 0.001$).

antigen-determination studies in schistosomiasis (Barsoum et al., 1991; Hassan et al., 1992). The treatment of urine samples with the alkaline solution gave higher CAA titres and concentrations than with the TCA treatment and also increased the sensitivity from 32% to 70% (Table 1). It seems most likely that this difference is due to the fact that part of the CAA present in urine is of low molecular weight, and therefore lost in the dialysis step of the TCA pretreatment. A major advantage of the new pretreatment is that the time-consuming dialysation and lyophilisation steps for concentration of the urine samples involved in the TCA pretreatment are no longer required.

CCA titres or concentrations of the urine samples remained essentially the same but an identical specificity (98%) was reached at 4-times lower cut-off levels (Tables 1 and 2). The slightly higher sensitivity (100%) which was observed for TCA pretreatment in comparison to alkaline pretreatment (96%) was only found in this group of individuals. When a larger group was tested, TCA pretreatment resulted in about 97% and alkaline pretreatment in 94% sensitivity (data not shown).

Additional advantages of this new technique are that the sample size needed, especially important for serum, can be reduced from 100 to 50 µl and that the technique is more suited for pretreatment of urine in the field, as no centrifugation or lyophilisation step is needed.

As detection of circulating antigens in urine, being a non-invasive technique, can be expected to be increasingly applied the next few years, the enhanced sensitivity

for CAA detection and the excellent CCA detectability with low non-specific reactivity, are further advantages of the alkaline pretreatment procedure.

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Chapter 2.9

Analysis of worm burden variation in human *Schistosoma mansoni* infections by determination of serum levels of circulating anodic antigen and circulating cathodic antigen.

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Analysis of Worm Burden Variation in Human *Schistosoma mansoni* Infections by Determination of Serum Levels of Circulating Anodic Antigen and Circulating Cathodic Antigen

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Serum circulating anodic antigen (CAA) and circulating cathodic antigen (CCA) concentrations, as a possible direct measure of worm burden, were compared with fecal egg counts in a heavily *Schistosoma mansoni*–infected population from Zaire to allow differentiation between worm loads and worm fecundity in relation to age and intensity of infection. Of the 517 subjects, 95% excreted eggs and 97% demonstrated circulating antigens. Fecal egg counts showed an age-related pattern characteristic for an area in which schistosomiasis is endemic with intense transmission levels. Regression analysis showed that antigen concentrations were strongly associated with egg counts. For CAA, but not for CCA, this relation was found to be nonlinear, which would be consistent with density-dependent fecundity or crowding. The trend was uniform for all age groups, which for this particular population indicated a genuine reduction of worm loads rather than reduced worm fecundity with age of the host.

In most areas in which *Schistosoma* organisms are endemic, prevalences and intensities of infection are higher in children than in adults. This characteristic age-related egg count pattern can be explained by reduced exposure or the development of protective immunity in adults (or both). Such "acquired immunity" could be directed against incoming larvae but possibly also against fecundity of established worms [1–5].

Thus far, studies on infection levels in humans are mainly based on counting eggs in excreta. Because individual fecal egg counts show strong day-to-day fluctuations, repeated examinations are required to get reliable quantitative data [6]. However, egg counts do not allow differentiation of effects of changes in worm loads from those in worm fecundity. In addition, egg excretion itself can be influenced by numerous host- and parasite-related factors [7]. For example, on the basis of data from the few available postmortem studies on worm loads and egg counts, it has been suggested that egg production is depressed in hosts with high worm loads [8]. However, this phenomenon of density-dependent fecundity or crowding is still a point of debate [9, 10].

In this study, we assessed worm burdens by measuring serum concentrations of two schistosome-specific circulating antigens, circulating anodic antigen (CAA) and circulating cathodic antigen (CCA) [11]. These gut-associated antigens are excreted in large quantities by the adult worms into the circulation of the host. Several experimental animal studies have shown a good correlation between worm loads and the levels of these antigens [12–14]. CAA and CCA can also be demonstrated in serum or urine of humans with *Schistosoma* infections by monoclonal antibody (MAb)–based sandwich ELISAs, showing a specificity of at least 98%, with sensitivities ranging from ~70% to 100% [15–18]. Several studies have proven the diagnostic value of these assays. Titers and concentrations are strongly correlated with egg counts and decline rapidly and strongly after successful chemotherapy [19–21]. However, most of these studies have been based on small or selected patient groups, which limits their epidemiologic interpretation.

In the present study, we investigated a large population across all age groups from an area where intense, year-round transmission of *Schistosoma mansoni* has been taking place for several decades. Regression analyses of antigen levels and egg counts were used to analyze worm burdens in relation to age, sex, and intensity of infection. Specific aims of the study were to determine whether circulating antigen levels, as a possible direct measure of worm burden, confirm the age-related pattern suggested by egg counts and to examine whether in the respective age groups the relation between circulating antigen (representing worm burdens) and egg counts provides evidence for density-dependent fecundity.

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Samples have been collected in consultation and full cooperation with both the local authorities and the persons concerned. Persons showing *Schistosoma* egg counts were treated with praziquantel according to the protocol current at the time of sample collection.

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Materials and Methods

Study population. The serum samples and parasitologic data came from the population of eight mining villages in Maniema (Kivu Province, Eastern Zaire) and were collected between 1978 and 1980. The area and the local epidemiology of schistosomiasis mansoni has been described [22, 23]. Fecal egg counts, expressed as eggs per gram of feces (epg), were based on two stool examinations, with an interval of a few weeks, each consisting of a duplicate 25-mg Kato-Katz smear [22, 24]. Serum samples were collected from a random selection of the population and stored at −70°C until use. Children <1 year of age were excluded. CAA and CCA levels were determined in serum samples of 517 persons (262 male, 255 female) with a median age of 30 years (range, 1–66).

Determination of circulating antigens. Sera were pretreated with trichloroacetic acid (TCA) to dissociate immune complexes and to remove interfering proteins [25]. CAA and CCA concentrations were determined by time-resolved immunofluorometric assays (TR-IFMAs), based on the highly specific MAb-based sandwich CAA and CCA ELISAs [15, 16]. In brief, CAA was captured onto MAb 120-1B10-A–coated microtitration plates (Maxisorp; Nunc, Roskilde, Denmark) and detected using biotin-labeled MAb 120-1B10-A. The lower detection limit of this assay was ~15 pg of CAA/mL. CCA was captured onto MAb 54-5C10-A–coated microtitration plates and detected using biotin-labeled MAb 8-3C10. The lower detection limit was ~60 pg of CCA/mL. After the biotin-labeled MAb step, streptavidin-europium conjugate (DELFIA; Pharmacia, Uppsala, Sweden) 1000 times diluted in DELFIA assay buffer was added. In both assays, all incubation steps except coating were shortened to 15 min by applying a shaking incubator system [26]. The last washing step was done with DELFIA washing buffer, after which enhancement solution (DELFIA) was added. Plates were read on a time-resolved fluorometer ARCUS-1234 (Pharmacia).

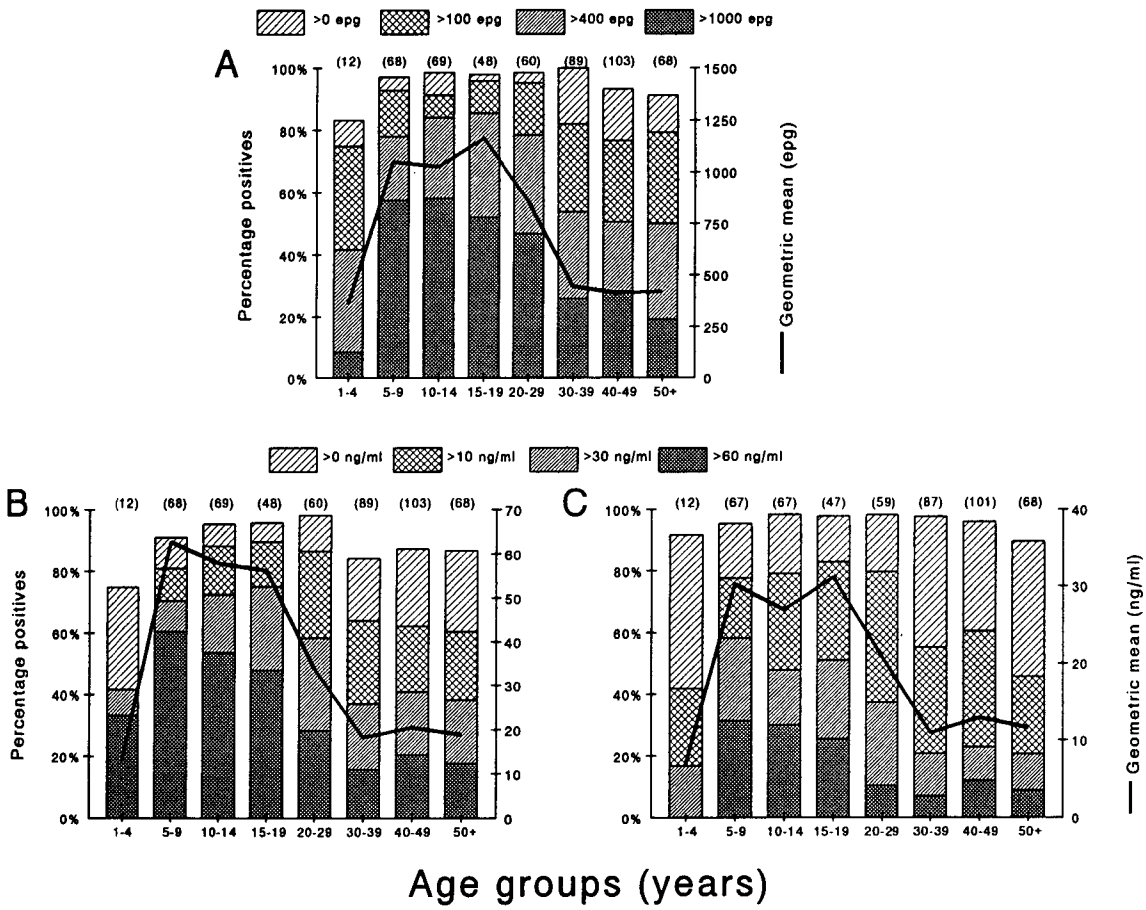

Figure 1. *S. mansoni* prevalence and intensity of infection, in different age classes, measured by fecal egg counts (**A**), serum CAA concentration (**B**), or serum CCA concentration (**C**). Bars represent percentages positive; lines represent geometric mean eggs per gram of feces (epg) or antigen levels (ng/mL) of those positive. Numbers of observations are shown at top of each bar.

Serum samples were tested in duplicate (final dilution, 8×) in assay buffer (0.2 *M* TRIS-HCl, pH 7.7, 0.15 *M* NaCl, 0.5% [wt/vol] bovine serum albumin) [27]. Serial dilutions of the TCA-soluble fraction of adult worm antigen (AWA-TCA) were assayed simultaneously on each plate to calculate CAA and CCA concentrations. AWA-TCA contains ~3% (wt/wt) CAA and ~3% CCA, as determined by using immunopurified antigen preparations [28, 29]. Samples were considered positive if measured counts were higher than the mean of 8 buffer controls plus 3 times the SD. If antigen concentrations fell outside the linear range (>250 ng/mL), samples were tested again at a higher dilution.

Data analysis. As circulating antigen levels and egg excretion showed skewed distributions, data were normalized by applying a $\log_{10}$ transformation on all positive values. Data were characterized by ranges, geometric means, and 95% confidence intervals (CI) of the geometric mean for all positive persons. The association between circulating antigen concentrations and egg counts was first examined by Pearson's correlation coefficient. The population was divided into 8 age categories and the variation of intensity of infection with age was calculated with one-way analysis of variance (*F* test).

To study the effect of age on the relationship between antigen level and egg excretion in more detail, we divided the population into 4 age groups for regression analysis. The regression lines are given by the equation $\log(\text{epg}) = \alpha + \beta\log(\text{CAA or CCA})$, with intercept α and slope β, or equivalently by taking the antilog: $\text{epg} = 10^{\alpha}(\text{CAA or CCA})^{\beta}$. Regarding antigen levels as a reflection of worm burden, a measure of fecundity can be introduced by calculating egg production per CAA (or CCA) unit: $F = \text{epg/CAA} = 10^{\alpha}(\text{CAA})^{\beta - 1}$. These equations clearly show that if $\beta = 1.0$, the relationship between epg and CAA (or CCA) will be linear, indicating a constant fecundity. If $\beta < 1.0$, the relationship between the two variables will be nonlinear, indicating a reduction in fecundity at high worm loads (antigen levels).

Although the variation in serum antigen concentrations is comparatively lower than the well-documented variation in egg counts, it still cannot be neglected [6, 7, 20, 22] (Polman K, personal communication). This will lead to an underestimation of the slopes, when using the standard least square regression analysis, with circulating antigen levels as the independent and egg counts as the dependent variable. To overcome this problem, we applied regression analysis according to Deming, which is an extension of the conventional linear regression analysis, allowing variation in the dependent but also in the independent variable [30, 31]. The required ratio of the within-variance of both parameters was calculated from the data of a previous study, in which both egg counts and serum CAA levels were measured three times in 20 schistosomiasis mansoni patients from the same area as the present study [20]. This ratio was 0.82, indeed indicating more variation in egg counts than in circulating antigen levels.

The few subjects negative for both egg excretion and circulating antigen determination were considered noninfected and left out of the regression analysis, although it is possible that some of them had light infections missed by both diagnostic methods. Persons negative for only one of these parameters were included. They were assumed to have light infections missed by one of the diagnostic methods [11, 32]. Exclusion of these cases could lead to a bias because of the difference in sensitivity of the respective methods. To properly include them in the analysis, a value equal to half the detection limit of the particular assay was assigned to persons concerned. Although the number of singly positive cases was not large, they might be essential for the outcome of the analysis, as they are on the outskirts of the regression line. For comparison and reference, regression analysis was also done with the data from persons positive for both tests only. A limited number of outlying points, >3 times the SD from the regression line, were rejected from the analysis. The effect of age was tested by covariance analysis. No corrections were made for age-related differences in blood volume, but likewise no corrections were made for the age-related daily stool production.

Results

S. mansoni eggs were found in the stools of 95% of the study population, 38% of which excreted >1000 epg. Positive egg counts ranged from 10 to 13,182 epg, with a geometric mean of 650 (95% CI = 578–731). No significant differences were found between male and female subjects.

Figure 1A shows the age–egg count pattern, which is characteristic for an area with intense *Schistosoma* transmission. The prevalence was already 83% in the youngest children, reached

Table 1. Percentage of subjects positive for circulating antigens and mean CAA and CCA concentration per egg count class.

	CAA			CCA		
Eggs/g of feces	No.	% positive	Concentration (95% CI)	No.	% positive	Concentration (95% CI)
0	26	35	3.6 (1.0–12.6)	25	76	2.4 (1.4–4.2)
1–100	47	72	4.9 (2.7–8.9)	46	91	5.1 (3.5–7.3)
101–400	106	91	14.6 (11.4–18.8)	104	96	9.6 (7.7–11.9)
401–1000	140	96	37.2 (31.0–44.8)	138	99	18.9 (15.6–22.8)
>1000	198	98	62.1 (53.5–72.1)	195	98	35.8 (30.6–41.9)
Total	517	90	31.3 (27.5–35.7)	508	96	16.4 (14.5–18.5)

NOTE. Concentration is geometric mean (ng/mL); CI, confidence interval.

99% in adolescents, and remained high in adults. The mean egg excretion showed a peak in the age groups of 5–19 years ($F = 8.9$, $n = 491$, $P < .001$).

Table 1 summarizes the results obtained with the two antigen assays, arranged by egg count class. Ninety percent of the total population was positive for CAA (concentration range, 0.056–514 ng/mL), 96% for CCA (range, 0.177–676 ng/mL), and 97% for at least one of these two antigens. Only 6 cases (1.2% of the study population) were negative in both antigen determination and egg counts.

For both CAA and CCA, the percentage of positive results and the mean circulating antigen level rose with increasing egg excretion (table 1). Although more persons were positive for CCA, CAA concentrations were generally higher, particularly in persons with high egg counts. CAA and CCA levels strongly correlated with each other ($r = .77$, $n = 452$, $P < .001$). Also, the correlation between egg excretion and circulating antigen level was significant, for both CAA ($r = .56$, $n = 457$, $P < .001$) and CCA ($r = .53$, $n = 469$, $P < .001$).

Antigen concentrations were divided into 4 categories, with about the same number of persons per category. Figure 1B and 1C show the age-related profiles for CAA and CCA, respectively. The age-related positivity rates mirrored the egg count pattern: >75% of young children, rising to at least 95% in adolescents, and remaining high in adults. The mean concentration of both antigens also showed a peak in the age groups of 5–19 years (CAA: $F = 10.1$, $n = 465$, $P < .001$; CCA: $F = 8.2$, $n = 487$, $P < .001$).

Figures 2 and 3 show scattergrams of the individual data for CAA and CCA, respectively, including the regression lines of the relation between log antigen level and log egg counts. For every given antigen concentration, an about equal distribution of egg counts can be noticed (and vice versa). Herewith, the statistical conditions of homoscedasticity and normality are

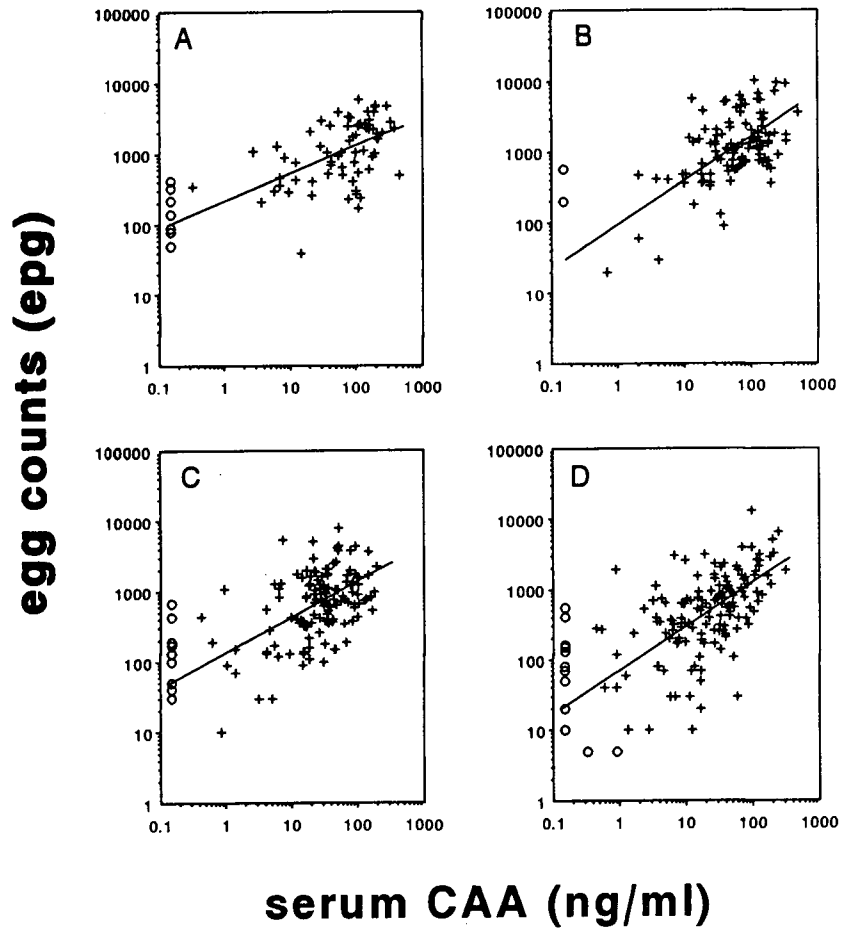

Figure 2. Egg counts per gram of feces (epg) plotted against serum CAA levels for 4 age groups: **A**, 1–9 years ($n = 76$); **B**, 10–19 years ($n = 112$); **C**, 20–39 years ($n = 140$); and **D**, ≥40 years ($n = 158$). Persons negative for both parameters ($n = 17$) and outliers ($n = 14$) were excluded. ○, persons negative for 1 of 2 parameters, in which case value was assigned representing half detection limit of particular assay. Each line represents outcome of regression analysis of all data points, given by $\log(epg) = \alpha + \beta\log(CAA)$, with slope ($\beta \pm$ 95% confidence interval) of: **A**, 0.39 ± 0.09; **B**, 0.62 ± 0.12; **C**, 0.51 ± 0.09; **D**, 0.63 ± 0.09.

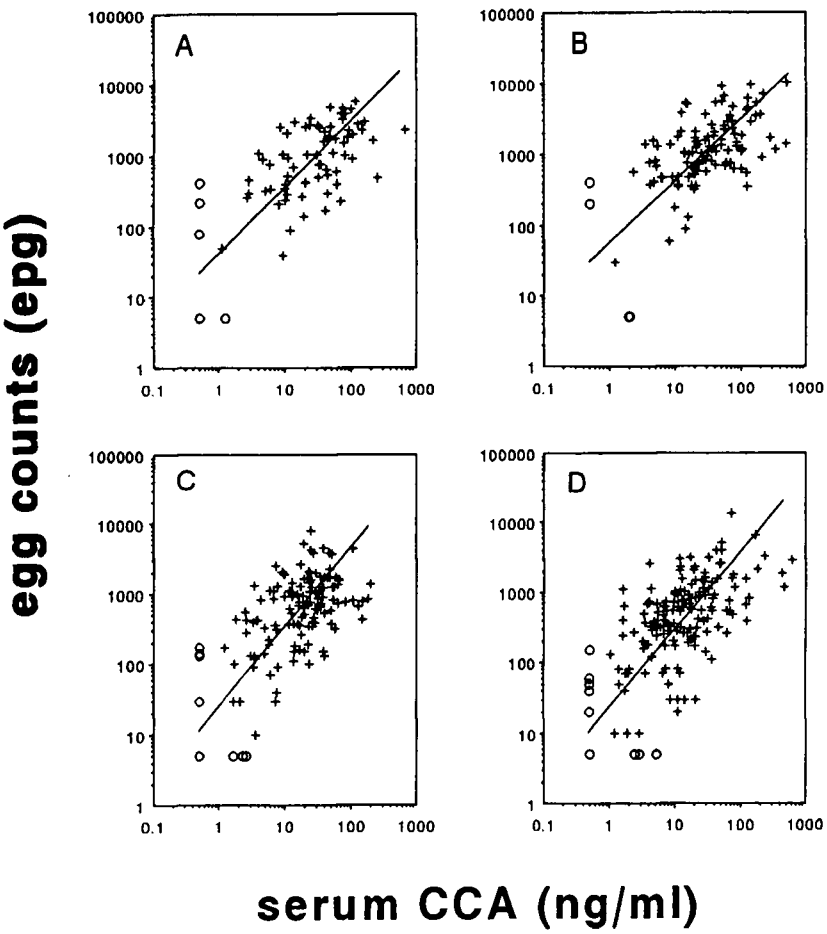

Figure 3. Egg counts per gram of feces (epg) plotted against serum CCA levels for 4 age groups: **A**. 1–9 years (n = 77); **B**. 10–19 years (n = 109); **C**. 20–39 years (n = 139); and **D**. ≥40 years (n = 158). Persons negative for both parameters (n = 6) and outliers (n = 19) were excluded. (See also figure 2.) Each line represents outcome of regression analysis of all data points, given by equation log(epg) = α + βlog(CCA), with slope (β ± 95% confidence interval) of: **A**. 0.93 ± 0.13; **B**. 0.86 ± 0.13; **C**. 1.14 ± 0.12; **D**. 1.10 ± 0.11.

reasonably fulfilled. The slopes of the regression lines are given in the legends of the figures. For CAA, slope β_{CAA} was significantly <1.0 in all 4 age classes, indicating that the egg counts do not increase as rapidly as the levels of CAA (figure 2). Although to a lesser extent, β_{CAA} was still significantly <1.0 when analyzing only the persons positive at both the parasitologic examination and the antigen assay. For CCA, the relationship between egg counts and antigen level was about linear, with β_{CCA} slightly >1.0 in those 20–39 years and slightly <1.0 in those 10–19 years (figure 3). Analyzing just the subjects positive by both tests, β_{CCA} equaled 1.0, except again for those 10–19 years old.

Covariance analysis revealed no significant influence of age on the relationship between circulating antigen concentrations and egg counts. For the total study population, β_{CAA} (±95% CI) was 0.56 (±0.05) and β_{CCA} was 1.04 (±0.08). No differences were found between male and female subjects.

Discussion

In the present study, CAA and CCA concentrations were determined by TR-IFMA, which compared with ELISA has the advantage of a longer linear range [33]. Sera could therefore be tested in one standard dilution instead of dilution series. The TR-IFMA for the detection of CCA was developed and applied here for the first time. For both antigens, the detection limit of the TR-IFMA was comparable to that of the standard ELISA, which means that we were not able to reproduce the detection limit of 20 pg of AWA-TCA/mL reported earlier for CAA [27].

Both CAA and CCA are heavily glycosylated and are extremely stable, as illustrated by the fact that CAA was still demonstrable in tissue of a 5000-year-old Egyptian mummy [34]. This allowed us to readdress serum samples that had been collected ~15 years before.

We found 97% of all egg excreters to be positive for CAA or CCA (or both) in serum. For the remaining 3% negative for both antigens, egg counts were generally <400 epg. These results confirm the diagnostic applicability of CAA and CCA determination, as shown previously [17, 19, 21, 35, 36].

The main goal of our study was to use circulating antigen quantification to allow a differentiation between worm reduction and reduced fecundity over age. As judged by the good correlation between the number of *S. mansoni* worms and CAA or CCA concentrations, as shown in animal studies, antigen levels supposedly reflect actual number of parasites [13, 14]. On the other hand, it is likely that the production and clearance of these antigens is affected by several partly host-related mechanisms. The production of CAA and CCA (as a reflection of worm metabolism) may be closely related to egg excretion, both mirroring the general well-being of the parasites. Also, the efficiency of the immune system to clear these antigens may be influenced by the age, health status, or sex of the host, although no differences between male and female subjects were found in this study. Even taking these limitations into account, estimation of worm burdens in humans by circulating antigen determination seems to be a valuable approach because it is, for the time being, the only alternative to the measurement of egg counts.

In general, the age-intensity curves of antigen levels resembled those of egg counts. Regression analysis according to Deming was applied to study the relation between these two variables in detail, with the emphasis on density-dependent fecundity or crowding. This phenomenon was first described by Medley and Anderson [8] from autopsy data of 65 persons with light to moderate *S. mansoni* infections. The population we studied here not only has a high number of participants but, more important, also represents a large range of infection intensities, including persons with extremely high egg counts. This implies that if density dependence is of any importance, it should certainly be demonstrable in a population as described here.

For CAA, we found slope β_{CAA} to be significantly <1.0, which may indicate a reduced fecundity at high infection levels. However, other explanations are still possible, such as increased egg retention in tissue. In contrast to CAA, the relationship between CCA levels and egg counts was almost linear. There are some indications that worm burdens are somewhat better reflected by the level of serum CAA than of CCA. For instance, CCA seems to be more efficiently cleared into the urine than CAA, as urine levels are much higher for CCA than for CAA [17, 18] (van Dam G, personal communication). In addition, specific IgM antibodies, as measured in an immunofluorescence assay against *Schistosoma* gut-associated antigens, are >90% directed against CCA, which indicates also that antibody-dependent clearance probably occurs much more efficiently for CCA than for CAA [37].

For both antigens, the slopes were calculated by using a ratio of the within-variance of the two variables based on a data set with comparably stable egg counts [20]. Future data sets will most likely show a lower ratio, automatically leading to a lower value of β. Therefore, more studies with repeated measurements of circulating antigen levels are needed to allow a thorough evaluation of their relative stability.

In conclusion, we found the relationship between CAA or CCA levels and egg counts to be consistent, independent of the age of the host. This suggests that in this heavily and chronically infected population, the decline in egg counts after adolescence purely results from a reduction in worm load and not from reduced worm fecundity, as suggested by others [1, 3, 38, 39]. For CAA, but not for CCA, our data are consistent with density-dependent fecundity. However, other mechanisms affecting production and clearance of the circulating antigens cannot be excluded, and to corroborate our findings, further research on these issues is needed.

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Chapter 2.10

Comparison of circulating antigen levels in two areas endemic for schistosomiasis mansoni indicates local differences in worm fecundity.

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Abstract

Serum levels of the adult worm schistosome antigens CAA (circulating anodic antigen) and CCA (circulating cathodic antigen) of two populations, both living in an area with extremely high transmission levels of *Schistosoma mansoni*, were compared. In one focus (Maniema, eastern Zaire) transmission had been established since several decades, while in the other focus (Ndombo, northern Senegal) transmission started only recently. While parasite egg counts were almost similar in the two populations, with a comparable age related distribution, serum levels of both CAA and CCA were approximately twice as high in the chronically exposed community in comparison to the recently exposed community. This difference in antigen levels was most pronounced for adolescents and adults. As CAA and CCA levels are assumed to be directly related to the number of parasites, these findings suggest comparatively lower worm burdens, and therefore higher worm fecundity, in the recently exposed community.

Introduction

Because of their intra-vascular localization, *Schistosoma mansoni* worm populations can not directly be quantified in infected humans. Usually, the dynamics of schistosome infections are studied by counting the number of parasite eggs in faeces or urine. However, egg excretion shows a considerable

day-to-day variation and light infections are easily missed.[1-3] Therefore, parasitological examinations need to be repeated several times to get an accurate impression of the individual intensity of infection. Besides, egg excretion itself is likely to be affected by several mechanisms and is even mentioned as a possible target of acquired immunity.[4,5]

Alternatively, detection of the *Schistosoma* specific circulating antigens CAA (circulating anodic antigen) and CCA (circulating cathodic antigen) allows sensitive and specific diagnosis of schistosomiasis in man.[6] Both antigens are glycoconjugates associated with the gut of the adult worm and are released by the parasite in large amounts into the circulation of the infected host, thus allowing detection in both serum and urine.[6,7] The good correlation between the levels of these antigens and the number of parasites, as shown in animal models, as well as the rapid clearance from the circulation following successful chemotherapy in humans, indicate that the levels of these antigens can also be interpreted as a reflection of current worm burdens in man.[8-11] A linear relation between worm burden and serum CAA level over a wide range of infection intensities was recently shown in a mouse model, although the lowest worm burden used in this study was still relatively high when extrapolated to the situation in man.[9] In this mouse study, suppression of the cellular immune system showed no change in serum CAA level.[9]

For these reasons, determination of circulating antigen levels, as a measure of worm burden, appears to be an interesting alternative to study infection patterns normally seen in endemic areas, i.e. a peak in faecal egg counts during adolescence, followed by a rapid decline in adults. This characteristic convex shape of intensity of infection has been interpreted as the slow acquisition (following years of infection) of partial protective immunity.[12] However, whether this immunity is directed against new incoming or existing parasites or against the worm fecundity, is still a point of debate.[13-15] The use of antigen detection in addition to egg counts would give valuable complementary information on the dynamics of worm populations as well as density-dependent mechanisms in different endemic settings.

Recently, we determined and analyzed serum CAA and CCA levels from Maniema, an area in eastern Zaire, endemic for *S. mansoni* since several decades.[16] No effect of age was found on the relationship between CAA or CCA concentrations and egg counts, indicating that the reduction in egg counts seen in adults after a peak during adolescence is caused by a genuine reduction in worm burden, in this particular population. At the same time, the relation between egg counts and serum CAA was found to be non-linear, i.e. at high worm loads (c.q. antigen levels) egg counts were not as high as expected in case of a linear relationship.[16] This agrees with the theory of density-dependent

fecundity, i.e. a decreased egg production by female worms in the case of high worm loads.[17]

The aim of the present study is to see whether history of transmission does influence the relationship between worm burden (c.q. antigen level) and egg output. According to the current hypothesis, resistance against schistosomiasis will develop only after many years of exposure and supposedly will still be absent in an area where transmission occurred only recently. Here we compared serum CAA and CCA data collected in Maniema with data collected in Ndombo, an area in northern Senegal with a recent outbreak of *S. mansoni* infection.[18] Due to the epidemic increase of schistosomiasis in this area, all individuals of Ndombo have probably become infected in a relatively short time frame. In this situation, therefore, history of infection is assumedly not related to age.[19,20] Despite differences in transmission history between the two populations, they show a striking similarity in faecal egg counts, ranging from low to extremely high levels. This makes comparison of circulating antigen levels even more interesting.

Materials and methods

Study populations

Parasitological data and serum circulating antigen levels were compared for two populations, both living in an area with intense transmission of *S. mansoni*. Both data sets were collected by scientists of our own laboratory. In general, the same protocols of parasitological examination and circulating antigen determination were used.

The first data set was collected in Maniema (eastern Zaire), an area where schistosomiasis had been endemic for several decades.[16] The area and the local epidemiology of schistosomiasis mansoni have previously been described.[21,22] Faecal egg counts were calculated from two stool examinations, each consisting of a duplicate 25 mg Kato-Katz smear.[21,23] Five hundred and eight cases, from which both CAA and CCA data were available, were used in the analysis.

The second data set has been collected in Ndombo (northern Senegal), a recently established focus where the first case of schistosomiasis was reported only a few years before sample collection.[18,24,25] To prevent any bias caused by differences with the Maniema data in the amount of stool examined, only those individuals were selected for further analysis from whom two stool samples were examined by a duplicate 25 mg Kato-Katz smear. In addition, both serum CAA and CCA levels had to be available. Compared to the original publications,[18,25] these selection procedures resulted in a reduction in the number of subjects

from 422 to 246. However, this selection did not lead to a statistically significant change in age, egg counts or antigen levels of the data sets.

Circulating antigen levels were determined by monoclonal antibody sandwich assays, as described by Deelder *et al.*[26] and De Jonge *et al.*,[27] with some minor modifications.[16,18] Both assays show a specificity of at least 98% and a sensitivity ranging from 50-100%, depending on the intensity of infection.[7,28,29] For accurate comparison between the two data sets and to again exclude any bias, serum samples were considered to be negative for CCA when showing a concentration <1.14 ng/ml, a cut-off level already used for the Ndombo data.[18]

Data analysis

The two data sets were analyzed as described in our previous paper.[16] In short: as circulating antigen levels and egg excretion show skewed distributions, data were normalized by applying a $\log_{10}$ transformation on all positive values. Data were characterized by ranges, geometric means and the 95% confidence intervals of the geometric mean (95%CI), for all positive individuals. Initially, the two populations were compared with the Student's t-test, while the association between circulating antigen concentrations and egg counts was examined by a Pearson's correlation test.

To study the effect of age on the relationship between antigen levels and egg excretion, the populations were divided into four age groups. Regression analysis according to Deming was applied, which is an extension of the conventional linear regression analysis, allowing variation in the dependent, but also in the independent variable.[30,31] The few cases which were negative for both egg excretion and circulating antigen determination were considered as non-infected and left out of the regression analysis. (For Ndombo: $n=1$ and $n=7$, for Maniema: $n=17$ and $n=10$, for CAA and CCA, respectively.) Individuals who were negative for only one of these parameters were included and a value equal to half the detection limit of the assay concerned was assigned to these subjects.[16] A limited number of outlying points, i.e. more than 3 times the standard deviation from the regression line, were rejected from the analysis. (For Ndombo: $n=5$ and $n=8$, for Maniema: $n=14$ and $n=19$, for CAA and CCA, respectively.) The effect of age was tested by covariance analysis.

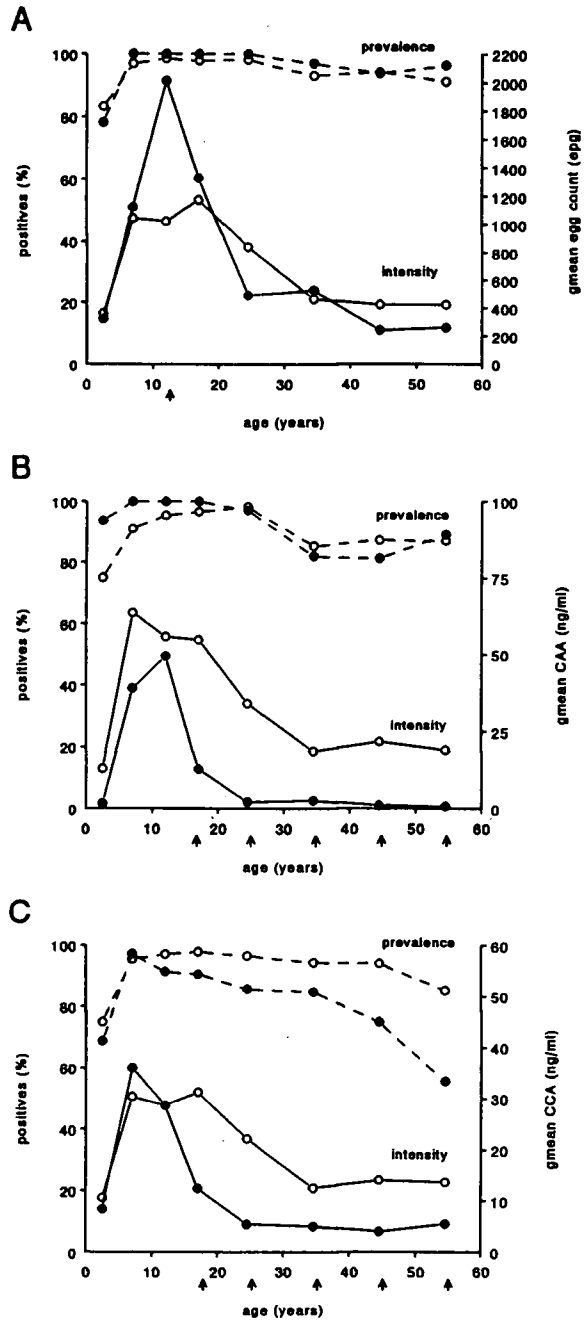

Figure 1. Comparison of *S. mansoni* prevalence (% positives) and intensity (geometric mean of positive values) of infection in two endemic areas in relation with age. Infections are measured by faecal egg counts (A), serum CAA concentration (B), or serum CCA concentration (C). The open circles represent data from Maniema (Zaire), the closed circles from Ndombo (Senegal).

Table 1: Comparison of two data sets, one from Maniema (Zaire), where intense, year-round transmission of *S. mansoni* has been taken place for several decades, the other from Ndombo (Senegal) where the transmission of schistosomiasis has been established only recently.

	Maniema	Ndombo	*P*
n	508	246	
Sex			
Males	51%	46%	n.s.
Age (Years)			
Range	1 - 66	1 - 77	
Median	30	18	<0.0001
Egg count (epg)			
Positive	95%	96%	n.s.
Range	10 - 13183	10 - 10328	
G.Mean	653	675	n.s.
CAA (ng/ml)			
Positive	90%	94%	n.s.
Range	0.06 - 514	0.02 - 339	
G.Mean	31.4	5.8	<0.0001
CCA (ng/ml)			
Positive	94%	84%	<0.0001
Range	1.19 - 676	1.29 - 2421	
G.mean	19.0	11.4	<0.0001

Abbreviations: *n*: number of subjects tested; epg: eggs per gram faeces; CAA: circulating anodic antigen, CCA: circulating cathodic antigen; G.mean: geometric mean of the positive values; n.s.: not significant.

Results

Table 1 summarizes and compares the data set of Maniema (eastern Zaire) with that of Ndombo (northern Senegal). All ages were represented in both groups, but on average the study population from Zaire was older. The number of infected individuals and the intensity of infection based on parasitological examination was extremely high in both communities. Although mean egg counts were remarkably similar between the two populations, both CAA and CCA levels were significantly lower in Senegal.

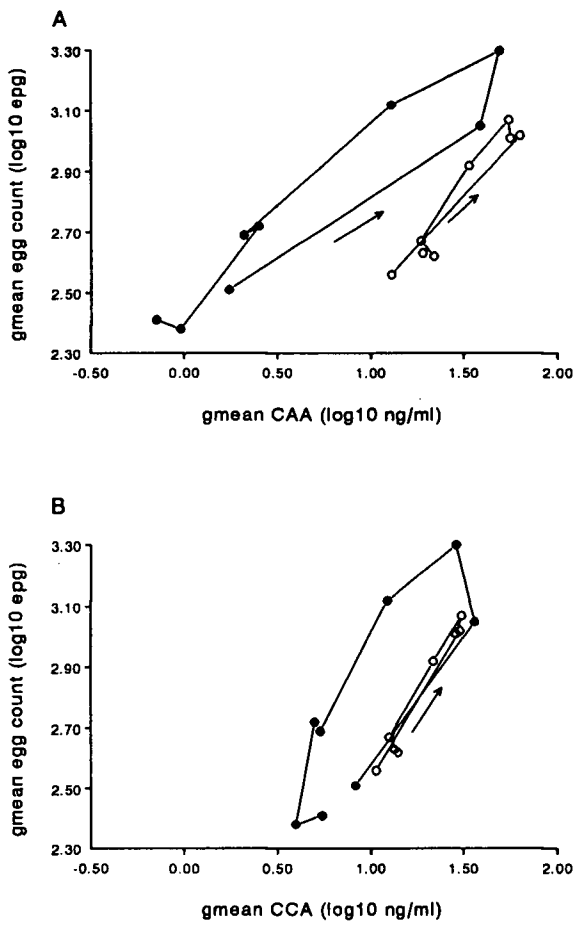

Figure 2. Relation between mean egg counts and serum CAA (A) or CCA (B) levels in eight consecutive age groups (as marked in figure 1) in Maniema (open circles) and Ndombo (closed circles). The direction of the arrow indicates an increase in age.

Table 2 summarizes CAA and CCA data in different egg output classes. In both populations, the percentage of positives as well as the level of antigen increased with increasing egg counts. Mean antigen levels were higher in Maniema than in Ndombo for all egg counts classes. CAA and CCA levels significantly correlated with each other and with egg counts in both populations ($P<0.0001$).

The age related distribution of egg counts shows a similar pattern for the two areas, except for a more pronounced peak in Ndombo in the age group of 10-14 years (figure 1). For CAA and CCA levels, the differences between the two populations are most pronounced above the age of 15 years. The sharp drop of CAA and CCA levels in adolescents from Senegal, suggests comparatively higher worm fecundity in adult hosts in this recent focus. This is also visualised by figure 2, clearly showing a shift in the relation between egg counts and antigen level with increase of age in the Senegalese population, while this is not the case for the population from Zaire.

To further analyze this hypothesis, regression analysis was performed in exactly the same manner as described before for the Maniema data.[16] Figure 3 and 4 show scattergrams of the individual data for CAA and CCA, for the four different age groups of the Ndombo population, respectively. Regression lines of the relation between log antigen levels and log egg counts are included in these figures. For comparison, these figures also depict the scattergrams of the Maniema population (3E and 4E). As no differences were found between the four age groups, all individuals of Maniema are represented together in one scattergram.[16]

Table 3 summarizes for the four age groups at Ndombo as well as for the total Maniema study group the intercept α and slope β of the regression lines given by the equation $\log(epg) = \alpha + \beta\log(CAA \text{ or } CCA)$. In contrast to the Maniema population, covariance analysis showed for the Ndombo population a significant difference between the four age groups in the relationship between egg counts and circulating antigen levels (data not shown). However, this effect of age seems to be a combination of several factors, not clearly attributable to one parameter. Most remarkable is the fact that above the age of 10 years, intercept α is significantly higher compared to the Maniema population, indicating a higher egg production for any given antigen level (c.q. worm burden). At the same time β_{CAA} is significantly lower than 1.0, meaning a non-linear relationship between egg counts and serum CAA levels, for the ages below 40, showing the lowest value at the age group of 10-19 years old. For CCA we see the same trends as for CAA, only β_{CCA} does not differ from 1.0 except for the 10-19 years old.

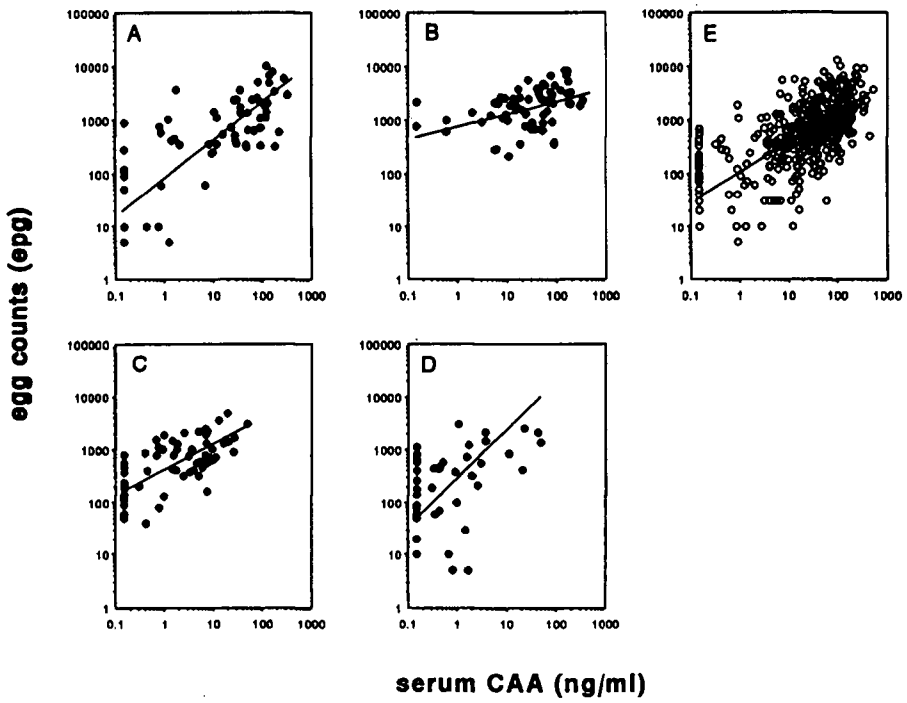

Figure 3. Egg counts plotted against serum CAA levels for 4 age groups in Ndombo: A = 1-9 years ($n = 66$); B = 10-19 years ($n = 66$); C = 20-39 years ($n = 65$); D = ≥40 years ($n = 43$) and all ages in Maniema: E ($n = 477$). Each line represents the outcome of regression analysis given by $\log(epg) = \alpha + \beta \log(CAA)$. The intercept α and slope β of these lines are given in table 3.

Discussion

The comparatively lower circulating antigen levels we found in the Ndombo population suggest a lower parasite burden, and therefore a higher worm fecundity, in this recent focus compared to the chronic situation. This finding is based on the assumption that actual worm loads are reflected by serum levels of CAA and CCA. However, it can not be ruled out that the production and/or clearance of these antigens is affected by several, in part host-related, mechanisms.[16] For example, the efficiency of the immune system to clear these antigens may be influenced by age or health status of the host, as well as by previous experience of infection. In this way, our findings could be interpreted as a relatively enhanced efficacy in adults from Ndombo to clear CAA and CCA from the circulation, probably due to the recent nature of their infections. The lack of difference between the two populations at younger age groups could then be explained by a general reduced clearance efficacy in children because of their still immature immune system.

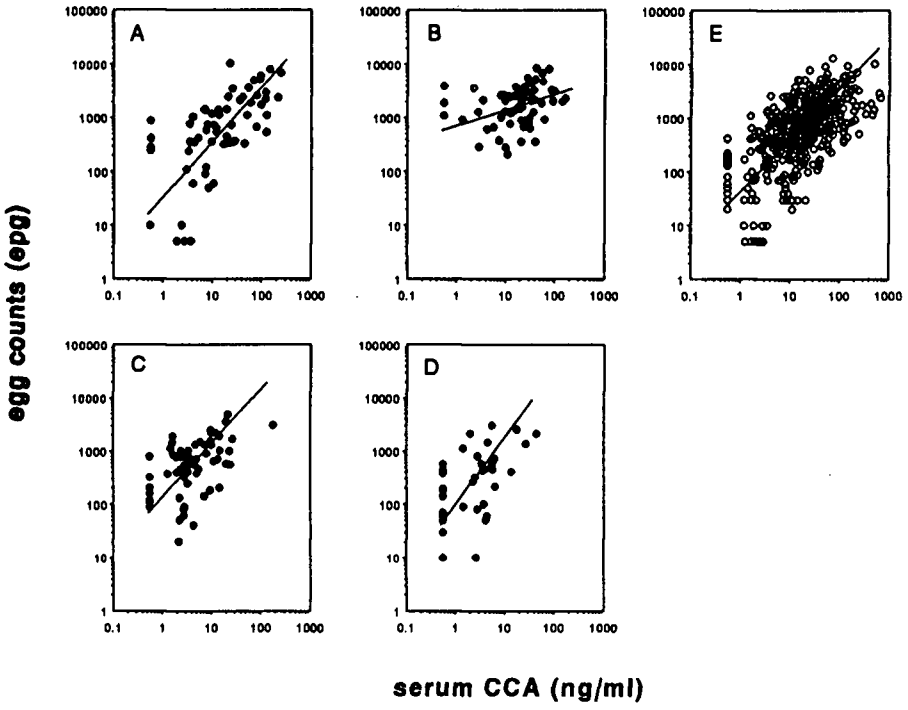

Figure 4. Egg counts plotted against serum CCA levels for 4 age groups in Ndombo: A = 1-9 years ($n = 63$); B = 10-19 years ($n = 66$); C = 20-39 years ($n = 65$); D = ≥40 years ($n = 37$) and all ages in Maniema: E ($n = 479$). Each line represents the outcome of regression analysis given by $\log(epg) = \alpha + \beta \log(CCA)$. The intercept α and slope β of these lines are given in table 3.

Indeed, an efficient renal clearance of CCA seems to be taking place in the Ndombo population, as urine levels of this antigen were relatively high compared to serum.[18] However, these high urine CCA levels were found at all ages and not only in adults. At the same time CAA urine levels were extremely low, indicating that the two antigens are cleared by different mechanisms.

Unfortunately, no urine samples were collected from the Maniema population. Determination of CAA and CCA in a chronically exposed community in Egypt showed a comparable serum/urine ratio in the levels of the two antigens as in the Ndombo population.[7] This argues against a major effect caused by renal clearance efficacy. On the other hand, the possible role of other clearance mechanisms, e.g. formation of immune complexes followed by clearance by Kupffer cells in the liver, can not be excluded.

Table 2: Percentage of subjects positive for circulating antigens and mean CAA and CCA concentration (ng/ml) per egg count class in two communities, chronically (Maniema) and recently (Ndombo) exposed to *S. mansoni*.

Maniema		CAA		CCA	
Egg counts (epg)	*n*	%Pos	G.mean	%Pos	G.mean
0	25	32	4.8	60	3.7
1-100	46	74	4.9	85	5.9
101-400	104	90	14.6	92	10.9
401-1000	138	96	36.3	98	19.3
1000 = >	195	97	62.5	98	35.8
Total	508	90	31.4	94	19.0

Ndombo		CAA		CCA	
Egg counts (epg)	*n*	%Pos	G.mean	%Pos	G.mean
0	10	90	0.4	30	2.7
1-100	29	79	0.4	72	3.5
101-400	41	88	2.5	71	7.0
401-1000	61	95	4.3	90	8.5
1000 = >	105	100	21.0	93	20.8
Total	246	94	5.8	84	11.4

Abbreviations: *n*: number of subjects tested; epg: eggs per gram faeces; CAA: circulating anodic antigen; CCA: circulating cathodic antigen; %Pos: percentage positives; G.mean: geometric mean of the positive values.

Table 3: Intercepts α and slopes β of the regression lines on the relationship between egg counts (epg) and circulating antigen level (CAA or CCA) in two communities, respectively chronically (Maniema) and recently (Ndombo) exposed to *S. mansoni*.

Group	Age	*n*	α	(95%CI)	β	(95%CI)
log(epg) = α + βlog(CAA)						
Maniema	all ages	477	2.1	(2.0 - 2.1)	0.56	(0.51-0.60)
Ndombo	1-9 years	66	1.9	(1.8 - 2.1)	0.72	(0.61-0.83)
	10-19 years	66	2.9	(2.7 - 3.1)	0.24	(0.13-0.36)
	20-39 years	65	2.6	(2.5 - 2.7)	0.48	(0.37-0.59)
	≥40 years	43	2.5	(2.3 - 2.7)	0.93	(0.68-1.18)
log (epg) = α + βlog(CCA)						
Maniema	all ages	479	1.6	(1.5 - 1.7)	0.98	(0.92-1.04)
Ndombo	1-9 years	63	1.5	(1.3 - 1.8)	1.03	(0.86-1.20)
	10-19 years	66	2.9	(2.7 - 3.1)	0.30	(0.14-0.46)
	20-39 years	65	2.1	(1.9 - 2.2)	1.02	(0.83-1.21)
	≥40 years	37	2.0	(1.8 - 2.1)	1.25	(0.99-1.52)

Abbreviations: *n*: number of subjects tested; epg: eggs per gram faeces; CAA: circulating anodic antigen, CCA: circulating cathodic antigen; 95%CI: 95% confidence interval.

Alternatively, the differences we found between the two populations could be explained by a difference in antigen production by the parasites themselves. This could be due to geographical strain differences, but it is also possible that due to the recent outbreak of schistosomiasis in the Ndombo population, a majority of infections consists of relatively young worms with a CAA and CCA production which has not yet reached its maximum level.[32]

In our previous study, we stated that we could not exclude a direct association between CAA/CCA production (as a reflection of worm metabolism) and egg excretion, both mirroring the general well-being of the parasites.[16] The differences we found in the current study between the two populations and between the different age groups in Ndombo, made this hypothesis less likely.

To gain a better understanding on the mechanisms of antigen production and clearance, further research on these topics is needed. Studies should preferably be undertaken in animal models imitating as closely as possible infection patterns seen in humans, as experimental infections differ in many aspects from the human situation, e.g. worm load in relation to body mass and the continuous exposure to reinfection.[33]

Another alternative explanation for our findings could be a difference in constitution of stool samples between the two populations. Indeed, faeces was very dehydrated in many subjects of the Ndombo population (Stelma, personal communication). This might have led to an overestimation of the actual egg excretion.[34]

Several animal studies have shown a significant correlation between the levels of CAA or CCA and the number of *Schistosoma* worms.[8-11] Assuming that circulating antigen levels indeed directly reflect actual worm loads in humans, our findings indicate that the egg production per worm pair in the recent established focus in Northern Senegal is relatively high in adolescents and adults compared to young children, as well as compared to the chronic situation of Zaire.

Recent studies have shown that immunization of animals with the anti-schistosome vaccine candidate Sm28-GST not only affected worm loads, but also resulted in a reduction of egg production and viability. This "anti-fecundity-immunity" was associated with an IgA response, inhibiting the enzymatic activity of this antigen.[35,36] Moreover, in human studies a correlation was demonstrated between anti-Sm28-GST IgA antibody levels and the amount of reinfection, suggesting that "acquired immunity" is partly directed against worm fecundity and the cause of the typical age-related egg count pattern as seen in chronically infected populations.[4,13,37]

Our results suggest that in case anti-fecundity immunity would be of any importance in *S. mansoni* infections, it seems to be more related to the history of transmission for the total population. However, we have no explanation for the fact that worm fecundity in young children from Ndombo seems to be relatively low compared to adults and is more comparable to the study population of Maniema.

The low levels of slope β we found in the group of 10-19 years old of the Ndombo population indicate a relatively strong density-dependent fecundity in this age group. This was also suggested in the original study describing the Ndombo circulating antigen data, by calculating the individual ratios between egg counts and antigen levels.[18] Determining the individual ratio between these two parameters seems to be the most straight forward method of analysis. However, there are several drawbacks to the use of ratios in statistical analysis, e.g. their highly skewed distribution and the undue weight given to small values of the denominator.[38]

Figure 3 and 4 clearly show that the strong density-dependent fecundity in the 10-19 years old, is mainly caused by a few subjects with low antigen levels (c.q. worm burdens) and relatively high egg counts, and not by a reduced egg production in individuals with high antigen levels (c.q. worm burdens).

In conclusion, we found significant differences in the relation between circulating antigen levels and egg counts after detailed comparison of two data sets, both collected from foci with comparable extremely high intensities of *S. mansoni* infections, but with a different history of transmission. The relatively lower serum levels of CAA and CCA in adolescents and adults living in Ndombo, suggest lower worm burdens, and consequently higher worm fecundity, in this area where transmission has taken place only recently. However, other mechanisms affecting production and clearance of these circulating antigens can not be excluded.

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Chapter 3

General discussion

Introduction

The work described in this thesis covers a period of several years of research on the determination of the circulating antigens CAA and CCA in human *Schistosoma* infections. Consequently, a relatively wide range of topics has been addressed throughout the different chapters. In this discussion, the drawbacks of parasitological examination will first be reviewed, as the diagnostic performance of circulating antigen determination has been compared with parasite egg counts throughout the thesis. The consequences of these shortcomings for the evaluation of circulating antigen determination will then be analyzed. Thereafter, several aspects of the detection of CAA and CCA in serum and urine will be discussed, including immunodiagnostic and epidemiological applications, and some technical improvements of the assays demonstrating these antigens. Data on other schistosome circulating antigens than CAA and CCA will be briefly reviewed.

Although CAA and CCA determination performed well in most studies, there are still several related questions which require more research before large scale application of the assays becomes feasible. In this chapter, the following additional topics will be discussed:
- how soon after the onset of infection can CAA and CCA be detected?
- how stable are the levels of CAA and CCA during infection?
- how soon after successful treatment are CAA and CCA cleared from the circulation?
- how is the production and/or clearance of these antigens influenced by parasite- and host-related mechanisms?
- how stable is the correlation between CAA and CCA levels and worm burden, can antigen determination actually be used to "count" the number of worms?

Such questions would appear to be most readily addressed by the use of animal models. However, these results should be interpreted with caution as experimental infections differ in many aspects from the human situation, e.g. worm load in relation to body mass and the continuous exposure to reinfection.[1]

Parasitological examination as the gold standard

The need to look at alternative methods for the diagnosis of schistosomiasis is driven, at least in part, by some of the shortcomings of the conventional parasitological techniques. In spite of these imperfections, parasitological diagnosis remains so far the gold standard in the evaluation of any alternative. A detailed knowledge of these shortcomings is therefore essential to properly interpret discrepancies when different methods are compared.

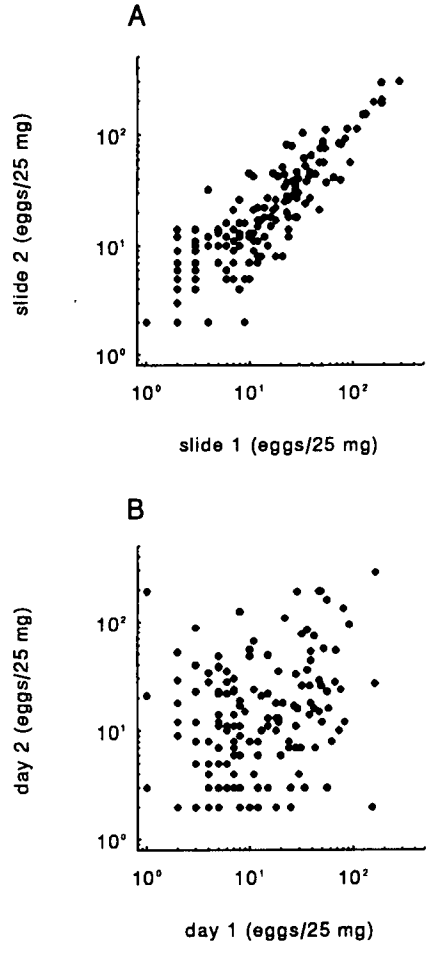

Figure 1. Reproducibility of *S. mansoni* egg counts of two Kato-Katz examinations (25 mg of faeces each), prepared from one and the same stool sample (A); or from two samples collected with an interval of a few weeks (B), in 205 individuals living in Maniema, Zaire (see also chapter 2.9). Data with kind permission of Dr. A.M. Polderman.

Problems of parasitological methods

The Kato-Katz method, extensively used for the parasitological diagnosis of intestinal schistosomiasis, is relatively easy to perform and gives satisfactory results in the diagnosis of moderate and heavy infections. However, a commonly noted problem is the large day-to-day variation in individual egg counts, as illustrated by Figure 1.[2-5] This may be explained by a variation in the number of eggs produced daily by female worms, or by a variation in the percentage of eggs retained in the host's tissue. In addition, differences in stool consistence, influenced by dietary intake or concurrent diseases, may also influence the number of eggs counted.[6] Subsequently, the final egg count appointed to each individual is depending on the number of stool samples examined. Whether the eggs of *S. mansoni* are homogeneously distributed within a stool sample, is still a point of debate.[7-10] The data illustrated by Figure 1 shows this intra-specimen variation to be relatively low compared to the day-today fluctuation. Anyhow, thorough mixing of the whole stool before taking a sample for examination, could minimize this type of variation. The contribution of variation between microscopists seems to be minor,[5] although this is disputed by others.[11]

Inherent to the Kato-Katz method is a sieving procedure, which concentrates the original sample up to 38% and may therefore lead to a slight overestimation of the real situation.[12] On the other hand, light infections can be easily missed in a single stool examination, due to the relatively small amount of faeces examined.[7,13-17] Even when stool examinations are repeated more than five times, additional positive cases can still be demonstrated.[18,19] Consequently, in low-intensity area's or during the follow-up after chemotherapy, prevalences are easily underestimated when applying the frequently used protocol of two or three stool examinations.[19] Based on a mathematical egg count model, De Vlas *et al.* recently developed a pocket chart which predicts the "true" prevalence of *S. mansoni* infection statistically, based on the data of a simple single stool survey.[19,20] This pocket chart could be of use for the evaluation of new diagnostic methods by providing, to some extent, a statistical gold standard (see also chapter 2.7).

In comparison, eggs can be more easily demonstrated in the urine filtration method used for the diagnosis of *S. haematobium* infection, because the volume of urine screened is relatively larger than the amount of stool examined in the Kato-Katz method.[12] In addition, urine does not contain fibre-like structures which may hamper the recognition of eggs. On the other hand, the number of eggs counted in the urine filtration method is strongly influenced by the protocol of sample collection, as most eggs are found around noon after physical exercise in combination with fluid intake, and in the last drops of miction.[21]

Besides the technical shortcomings of parasitological examination, there are also biological factors which may influence the number of eggs counted. It has been suggested, e.g., that the egg production per female may be reduced when the number of adult worms is very high (a process also known as "density dependent fecundity" or "crowding")[22] or by development of anti-fecundity immunity.[23] These factors mainly have implications on the analysis of data if circulating antigen levels are used for the assessment of worm fecundity (see also chapter 2.9 and 2.10).

In epidemiological studies, mean parasite egg counts are often used to describe intensity of infection at the population level. Usually, geometric means are calculated, because of the negative binomial distribution of the egg counts. This requires a (logarithmic) transformation of the data, which is not possible for zero egg counts. To overcome this problem an additional x + 1 transformation may be performed, or the zero egg counts may be left out. However, both methods will result in considerable differences in the outcome, which implies that the prevalence of schistosomiasis in a population influences the calculated mean intensity of infection.[5,24] Infection levels of a community are also often described by dividing the population in categories, based on their parasite egg counts. However, due to the day-to-day fluctuations described above, individuals are often incorrectly classified when egg counts have been calculated from only one stool or urine examination.[11,18]

Comparison with circulating antigen determination

The shortcomings described above should be taken into account when parasitological examination is used as a yardstick to evaluate the diagnostic performance of other tests. For example, a positive result for CAA in a case where no eggs have been demonstrated, could indicate either false positivity of the CAA assay or false negativity of the parasitological examination. Also the strength of association between egg counts and antigen levels is dependent on how thoroughly egg counts have been determined. This is illustrated by figure 2, showing an improved correlation between serum CAA level and egg counts calculated from three stool samples, as compared to one.

Most studies, including this thesis, found the diagnostic performance of CAA and CCA determination to be at least comparable with repeated parasitological examination, with sensitivity and reproducibility in the same order of magnitude. In contrast to that, parasitological examination was found to perform better with regard to fluctuation in a recent study in which CAA and CCA levels were determined in urine of *S. haematobium* subjects from Gabon (Van Etten *et al.*, manuscript in preparation). In this study, the procedure of urine collection

and parasitological examination were exceptionally well-standerdized. This confirms the importance of the followed parasitological procedure.

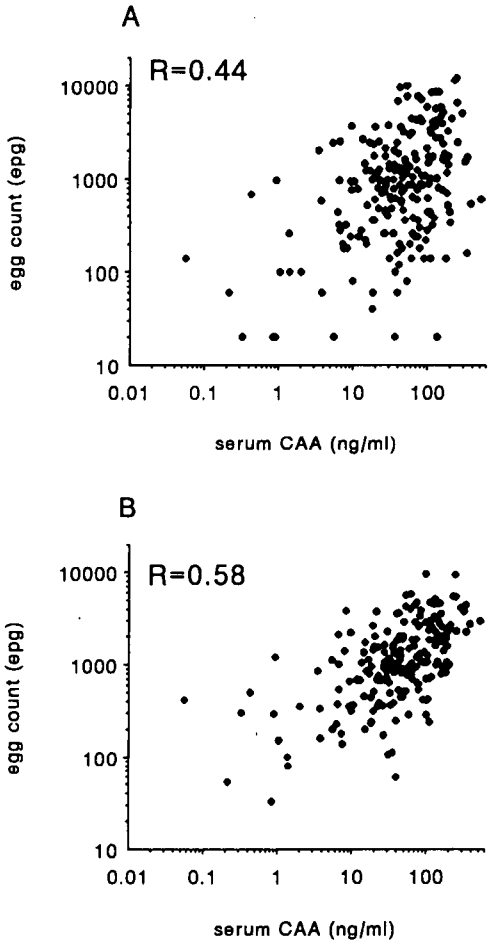

Figure 2. Correlation between serum CAA concentrations and epg (*S. mansoni* eggs per gram faeces) in 215 individuals living in Maniema, Zaire (see also chapter 2.9). Egg counts were calculated based on a duplo Kato-Katz examination prepared from one stool sample (50 mg of faeces in total) (A); or from three stool samples (150 mg of faeces in total) (B). R = correlation coefficient. Data with kind permission of Dr. A.M. Polderman.

The comparison between the diagnostic performance of circulating antigen determination and parasitological examination has been a recurrent item during this thesis. Four studies (chapters 2.1 and 2.3-2.5) have dealt with a group of hospitalized Egyptian schistosomiasis patients which all had a parasitologically proven *Schistosoma* infection, based on three duplicate stool examinations. CAA and CCA could be demonstrated in serum and urine in 82-88% of these cases, and although the study population was small, CAA and CCA levels

significantly correlated with egg counts. Following chemotherapy, just two consecutive stool samples were examined, while at that stage the intensity of infection, if still present, was found to be very low (chapters 2.3-2.5). In contrast to the pre-treatment situation, more active infections were demonstrated after treatment by circulating antigen determination, compared to the parasitological examination. This again illustrates the relevance of the followed parasitological procedure, when comparing these two diagnostic methods.

The importance of the number of stool examinations was also recently shown in a study on the epidemiological usefulness of circulating antigen determination in a recent *S. mansoni* focus in Northern Senegal.[25] This study showed substantially higher sensitivities of the serum CAA and urine CCA assay as compared to a single (25 mg) stool examination. Sensitivities were found to be similar to a two times duplicate 25 mg stool examination.

Two chapters of this thesis have dealt in particular with light infections. The first concerned an epidemiological area with low transmission levels in Surinam (chapter 2.7), the other a clinical examination of a group of Dutch tourists who got exposed once during a visit to an endemic area in Mali (chapter 2.8). In both studies the results of circulating antigen determination were comparable with the parasitological examination. In the Surinam study, a single circulating antigen assay (e.g. CCA urine test) showed the same number of positives as the parasitological screening, (of two duplicate stool samples), but both techniques missed a substantial part of the (lightly) infected cases. In the group of Dutch tourists, exceptionally thorough parasitological examination took place, including sedimentation techniques. Circulating antigen examination could only identify the same number of infected cases if the results of both CAA and CCA (in serum) were combined. No correlations were found between the levels of antigen and the parasitological findings. In both studies, CAA and CCA concentrations were often only slightly above the cut-off level of the assay. Notwithstanding the fact that both circulating antigen assays show a very high specificity, this makes application of CAA and CCA determination for routine diagnosis of lightly infected individuals questionable. Although the assays currently used can demonstrate already extremely low antigen concentrations, further improvement of the detection limits may benefit the applicability of CAA and CCA determination in these particular situations (Deelder *et al.*, unpublished data).

Also when comparing the levels of circulating antigens between different endemic areas, egg counts are often used as a reference. Again, also in these type of studies it is of great importance to standardize not only antigen

detection itself, but also the procedure of parasitological examination. In chapters 2.9 and 2.10 we excluded those individuals from which only one stool sample was examined. In case more than two examinations were performed, we only used the data of the two last before chemotherapy. On the other hand, this procedure may introduce a selection bias, as the compliance during the course of the study may differ between certain age groups or sexes.

Comparison of circulating antigen levels between selected individuals belonging to the same egg count category, should be performed with caution. By using a mathematical approach, it was recently suggested that the actual number of *S. mansoni* worms in individuals with the same egg count may differ, depending on the characteristics of the endemic situation.[26] Indeed, we found considerably lower levels of CAA and CCA in *Schistosoma* infected cases excreting less than 100 epg and living in a low transmission area in Surinam (chapter 2.6), compared to those living in an endemic area in Egypt (chapter 2.1). Although regional differences in both production and clearance of these antigens can not be excluded, these findings support the epidemiological model suggested by Gryseels and De Vlas.[26]

Determination of circulating antigens

While a large amount of data is available on parasitological examination, comparatively less is known about circulating antigen determination. Partly this is due to the fact that, although schistosome circulating antigens were described for the first time in 1958,[27,28] highly sensitive assays, which made the detection of these antigens in infected humans feasible, only became available about ten years ago.

In addition to CAA and CCA, a number of other circulating antigens, associated with different life cycle stages of the parasite, were studied for their diagnostic application. Table 1 summarizes recently published data on the detection of these antigens. The number of studies on humans is limited and only a few of these antigens have been more extensively characterized and tested in an animal model.

Some of these antigens may provide information complementary to data on antigens associated with adult worms. For example, circulating cercarial antigens (e.g. CFT) may have an application for the diagnosis of acute schistosomiasis. However, following some initial work on infected mice and pooled serum of chronically infected schistosomiasis patients (see table 1), no further human studies have been published. Detection of circulating egg antigen seems

to be promising and may be used for the determination of tissue egg burdens. It may provide information on egg-induced pathology, or may be used to measure worm-fecundity.

Table 1. Determination of circulating antigens other than CAA and CCA for the diagnosis of *Schistosoma* infection in humans.

Name	Associated with: Characterization:	Human studies showed:	Animal studies showed:	Ref:
CFT	- cercariae - 41 KDa - hydrophobic polypeptide	- detectable in serum chronic schistosomiasis (limited study) - *S.m./S.h./S.j.* - low specificity	- detectable in serum mice 3 days after exposure to 250 cercariae	29 30
PEA	- epithelium adult worm - large amounts in eggs - polysaccharide	- detectable in urine - *S.m./S.h./S.i.* - sensitivity much higher than parasitological examination for *S.m.* and *S.h.*, but not for *S.i.* - reduction after chemotherapy	- decrease in urine of mice following chemotherapy	31 15 32 33
CSA	- adult worm	- detectable in serum *S.m.*; in 61% of egg excreters - negative 3 months after successful chemotherapy	n.d.	34
CSA	- dominantly in *S.m.* - eggs - other stages(?) - glycoprotein with carbohydrate epitope	- detectable in serum *S.m.*; in 78% of egg excreters - reduction after chemotherapy - 79% of egg negatives also antigen negative 3 months after chemotherapy	n.d.	35

CSEA	- dominantly in *S.m.* - eggs - also present in other stages - carbohydrate epitope	- detectable in serum and urine *S.m.*, in 84% of egg excreters - correlation with egg excretion	- detectable in serum mice 8 to 9 weeks after exposure - not detectable in mice after uni-sexual infection	36 37
Sj70	- dominantly in *S.j.* - eggs - also present in other stages - protein - hsp70 associated	- detectable in serum *S.j.*, in 97% off egg excreters - 94% of egg negatives also antigen negative 6 to 12 months after chemotherapy	- detectable in serum mice 4 weeks after exposure with 30 cercariae - no correlation with worm burden - decrease following chemotherapy	38 39
Sj31/31	- adult worm gut - protein - Cathepsin B - haemaglobinase	- detectable in serum *S.j.*, in 68% of egg excreters	n.d.	40
Sj26	- adult worm - protein - glutathion-S-trans-ferase	- detectable in serum *S.j.* - association with resistance to reinfection	n.d.	41

Abbreviations: CFT = cercarial freeze-thaw preparation; PEA = polysaccharide excreted antigen; CSA = circulating schistosomal antigen; CSEA = circulating schistosomal egg antigen; *S.m.* = *S. mansoni*; *S.h.* = *S. haematobium*; *S.i.* = *S. intercalatum*; *S.j.* = *S. japonicum*; Ref = reference number; n.d. = not done.

Diagnostic performance of CAA and CCA

Of all circulating antigens described, most research so far has been performed on CAA and CCA. Several aspects of the diagnostic potential of these antigens have been discussed in previous papers and have been recently extensively reviewed by Deelder *et al.*.[42]

Initially, studies were mainly focused on the determination of CAA in serum.[43] The work described in this thesis further evaluates the detection of both CAA and CCA in serum and urine. The sensitivity of the two antigen

assays in the two body fluids was found to be similar in a group of Egyptian schistosomiasis patients, ranging from 82-88% (chapter 2.1). In contrast, two other studies showed a comparatively low sensitivity of the urine CAA assay (chapters 2.6 and 2.8). Besides the fact that levels of CAA in urine are very low in general, this could also be explained by the adaption of the cut-off level of the assay, which was needed after testing a larger group of negative controls (chapter 2.8).

Undoubtedly, the detection of antigens in urine would be preferable, as it involves a non-invasive method of sample collection. Although good results were obtained with the demonstration of CCA in urine in *S. mansoni* and *S. intercalatum* infections,[44,45] the application of this assay for the diagnosis of *S. haematobium* is still controversial.[46,47] For *S. japonicum* infections, no studies on the diagnostic performance of the urine CCA data have been published yet.

Using serum samples, there are no major differences between the diagnostic performance of CAA and CCA determination. Some studies found the CCA assay to be slightly more sensitive than the CAA assay, while other studies showed the opposite. Still, if a choice has to be made between the two assays, the use of CAA determination may be preferable. Both assays are highly specific (>98%), but the specificity of the CAA assay is comparatively slightly higher (chapter 2.8). Moreover, CAA contains unique polysaccharide structures,[48] while CCA partly consists of poly lewis x trisaccharides, which may be cross reactive with human inflammatory markers.[49,50]

Improvement of the assays

Although not the main goal of our study, we also performed some research on the technical improvement of the assays demonstrating CAA and CCA. Many other studies focused on the use of antigen detecting assays under field conditions.[51,52] Our objectives were (i) to increase sensitivity while maintaining high specificity and (ii) to simplify the assay procedures in such a way that larger amounts of samples could be tested simultaneously.

Increased sensitivity could be achieved by parallel testing and combining the circulating antigen data (chapter 2.1), which was possible because of the high specificity (98-100%) of the individual assays. As the different studies did not reveal one of the antigen assays to have the best diagnostic performance, there also seems to be no unique combination of antigen tests which universally leads to the best results. This method of parallel testing seems to be specially useful in identifying individuals with light infections, but it is also an advantageous approach for those cases with moderate to high egg excretion, who, for unclear reasons, are negative for one or more of the antigen assays.

Parallel testing and combining antigen data is a qualitative interpretation of

the available quantitative information. This does not have to be disadvantageous. Analogous to parasitological examination, the need for quantitative data for the diagnosis of individual cases is controversial, as the relation between intensity of infection and the development of pathology is still equivocal. Although a logical approach, simultaneous detection of CAA and CCA in one assay did not result in the expected increase in sensitivity, due to increased cut-off levels (chapter 2.2). However, simultaneous determination of two or more antigens may still be a potentially useful approach for other circulating antigens, e.g. egg antigens.

In an attempt to increase the sensitivity of the available assays, research was also concentrated on the evaluation and further development of TR-IFMA's. Three different methods were compared: direct labelling of the MAb with europium, application of FITC-anti-FITC/europium and of biotin-streptavidin/europium. Best results were obtained with the last approach, but a strong improvement of the detection limit, as previously reported for CAA,[53] could not be reproduced (Van Lieshout *et al.*, unpublished data). Still, linear ranges were significantly larger than those of the sandwich ELISA. This allowed determination of serum or urine samples in one dilution only, which, in contrast to serial dilutions, makes a relatively high through-put of samples (200/day) feasible. Also due to the longer linear range, antigen levels could be more easily expressed in concentrations instead of titres, which has statistical advantages. It allowed better standardization of antigen levels and made comparative studies achievable (see chapters 2.9 and 2.10). Alternatively, an increased linear range could also be accomplished with the ELISA by kinetically reading the absorbances within 2 hours after addition of the alkaline phosphatase substrate (Van Dam *et al.*, personal communication).

Recent infections

In experimental *Schistosoma* infections, CAA and CCA were found to be detectable for the first time, 2 to 5 weeks after exposure (see also table 1 of chapter 1). This is in accordance with the time period found in an unique human study, where the exact date of exposure was known (chapter 2.8). There, CAA and CCA could be determined in serum after 4 and 5 weeks, respectively. However, intensities of infection were extremely low in this group of Dutch travellers. A shorter period might be expected in individuals who live continuously in an endemic area and may be more intensively exposed.

Fluctuation of antigen levels

Like for parasitological diagnosis, levels of CAA and CCA in serum or urine are generally based on the determination of a single specimen. Nevertheless, for

reliable diagnosis and appropriate interpretation of quantitative data, it is important to know whether antigen levels are stable during an active infection. Studying day-to-day fluctuation of serum CAA levels, De Jonge *et al.* showed antigen fluctuation to be lower than *S. mansoni* egg excretion.[54] This was confirmed in a recent study on serum CAA and CCA levels in *S. mansoni* infected individuals from Burundi (Polman *et al.*, unpublished data).

In addition to serum, levels of CAA and CCA in urine, which are more likely to be influenced by physiological processes, e.g. the volume of urine daily produced, have extensively been studied in *S. mansoni* infected individuals. Seven urine and faeces samples were collected from 60 cases during a period of four weeks. Although both antigen levels and egg output fluctuated, urine CCA levels, but not CAA levels, were found to be more stable than faecal egg counts.[55] We found, although tested in a very small population, both CAA and CCA urine levels to be slightly more stable than egg counts (figure 3), which is in contrast with the CAA data of the previous study.[55]

Also experimental studies on the fluctuation of antigen levels have been performed. Goats infected with *S. curassoni* and followed for 30 weeks, showed substantial fluctuations in serum CAA and CCA levels, although slightly less than those of egg counts (Van Lieshout *et al.*, unpublished data). A study on cattle naturally exposed to *S. mattheei* infection and followed for 1.5 year, suggested a seasonal variation in CAA levels. Serum concentrations of this antigen increased during low transmission season, which is also a period of physical and nutritional stress for the cattle. This may imply a variation in antigen clearance rate related to the physiological condition of the host.[56]

In conclusion, although day-to-day variation in CAA and CCA levels is relatively less compared to egg counts, it can not be neglected. From a primarily diagnostic point of view, repeated sample collection may increase the chance of demonstrating CAA or CCA in lightly infected individuals, as also shown in chapter 2.7.

Follow-up after chemotherapy

As circulating antigens are a reflection of an ongoing active infection, they are a powerful tool for the assessment of cure following chemotherapy. Using CAA and CCA determination for this purpose requires the same approach as the diagnosis of light infections, i.e. the sensitivity of the assays detecting these antigens is of great importance. On the other hand, the need to diagnose and completely cure every infection, even those with very low intensity, is not absolute. Whether significant pathology will only develop in individuals with high egg counts, and whether light infections contribute to the transmission and reinfection of a treated population, are still points of debate.[57,58]

As discussed above, the sensitivity of the current CAA and CCA assays can be increased by parallel testing and combining different antigen data. This approach has also been followed for the assessment of cure after treatment with praziquantel, which indeed resulted in better diagnostic performance (chapter 2.5).

Based on parasitological examination, it can be very difficult to distinguish reinfection from failure of cure, especially in areas where transmission levels are high. Viable eggs can still be demonstrated in the excreta for several weeks following chemotherapy, in particular for *S. haematobium* infections. A rapid decrease of antigen levels following successful treatment, would make circulating antigen determination an interesting alternative, as it takes several weeks before CAA and CCA can be detected after the establishment of a new infection (chapter 2.7).

Indeed, almost all tested individuals were found to be negative for CAA in serum or CCA in serum and urine, two to three weeks after treatment with comparatively high doses of praziquantel (chapter 2.3 and 2.4).[54] However, after treatment with the standard dose of 40 mg praziquantel/kg, circulating antigens could still be demonstrated after 6 weeks in two third of the hospitalized patients (chapter 2.5). This indicates that the high cure rates found in a majority of the epidemiological studies,[59] give a too optimistic view, as probably many light infections have been missed by the parasitological examination. An alternative explanation would be that 40 mg praziquantel/kg does not completely kill the adult worms, but only immobilizes them temporarily. As a consequence, this may result in a reduced egg production while circulating antigen excretion still continues.

In conclusion, determination of CAA and CCA has proven its value for the assessment of cure and for the comparison of different treatment protocols. However, in areas with extremely high transmission levels and following treatment with the standard dose of 40 mg/kg praziquantel, it would appear that even these antigens can not be used to distinguish failure of cure from reinfection.

Production and clearance mechanisms

CAA and CCA are excreted by the parasite immediately after transformation to schistosomula, as shown by *in vitro* cultures. During the first days, the production levels of CAA and CCA are similar, but after one week of culture more CCA is excreted.[60] A relatively higher CCA production was also shown during culture of adult worms, following perfusion of infected mice or hamsters.[60,61] Both antigens were found to be excreted mainly by female

worms.[60] Whether cultured worms behave metabolically similar to the natural situation is questionable.[61]

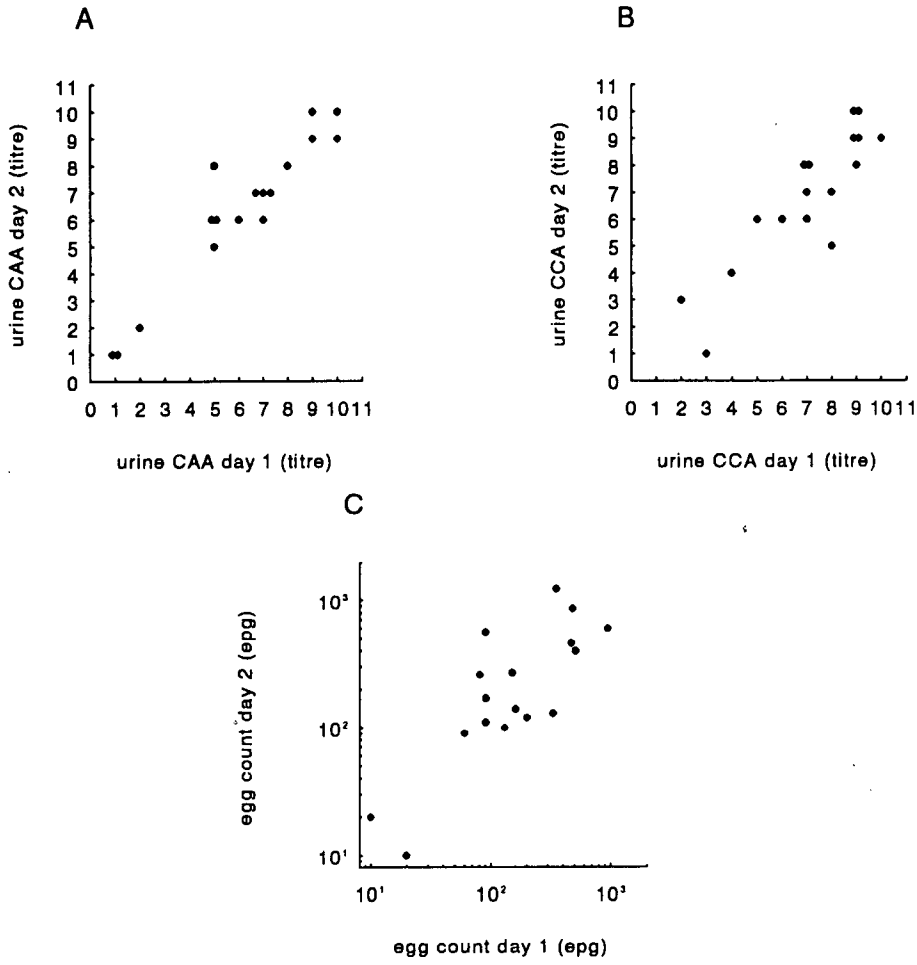

Figure 3. Reproducibility of CAA levels in urine (A), CCA in urine (B), or faecal egg counts (C) measured on two consecutive days, in 17 Egyptian schistosomiasis patients (see also chapters 2.3 and 2.4). epg = *S. mansoni* eggs per gram faeces.

Clearance of CAA and CCA can be explained by three different processes, which are influenced either by the properties of the antigen itself or by the host's immune responses or a combination of these two: (i) direct binding of the antigens to receptors on phagocytosing cells; (ii) filtration via kidney glomeruli into urine; and (iii) formation of immune complexes by binding to antibodies. The location where immune complexes (IC) are formed and the rate of clearance are

predominantly determined by the ratio between antigen and antibody concentration. Excess of antigen results in small IC, which do not fix complement or initiate an inflammatory process. They are not rapidly removed from the circulation.[62]

Nash studied the clearance rate of CAA in mice by injecting radioisotope labelled CAA, with or without specific antibodies. In non-infected control mice it was observed that the antibody mediated clearance mechanisms were much faster than the direct clearance of this antigen.[63] In addition, CAA was cleared faster in lightly infected animals than in heavily infected animals, which was related to the level of specific antibodies. As there was no difference in the clearance of pre-formed complexes, this indicates that the difference between the two infected groups was not caused by blocking of the receptors responsible for the uptake of the IC.[63] Indeed, it was found that lightly infected animals formed larger, and therefore more rapidly cleared, IC than the heavily infected animals.[64]

Kestens *et al.* also found that the clearance rate was determined by the size of the IC.[65] They showed this by injection of *in vitro* prepared IC, made of worm antigen in combination with radiolabelled anti-CAA MAb. IC were cleared at a significantly lower rate in infected mice compared to controls, indicating impaired uptake by the liver. In contrast to the data of Nash, larger size IC were mainly found in the group of heavily infected animals, while in lightly infected animals only small IC and free antibodies could be demonstrated.[65]

Less research has been done on the clearance mechanisms of CCA. In contrast to the results with CAA, Kestens *et al.* found for CCA similar clearance rates in infected and control mice after injection of *in vitro* prepared IC of different sizes, made of worm antigen in combination with radiolabelled anti-CCA MAb.[66] Again large IC were found to be most rapidly cleared and the tissue distribution of the cleared IC was again depending on the size.[66] Nash showed that PSAP, a glycoprotein with many similarities to CCA, but clearly distinct,[67] was cleared from the circulation not by a antibody mediated, but by a Galactose-receptor-mediated mechanism.[68]

All these studies, based on the injection of labelled material, emphasize the sensitive balance between antibody and antigen levels, which again may be influenced by the experimental set-up itself.

Very recently, several studies have been published which simply follow the kinetics of CAA and CCA during a certain time period of an experimental infection, by quantifying the levels of these antigens using the MAb-based assays. This approach has the disadvantage that the mechanisms regulating production and clearance of these antigens are very difficult to disentangle.

Van Dam and colleagues showed that after infection of mice with 1000 *S. mansoni* cercariae, in serum CCA could be detected first (starting from day 16), followed by CAA (starting from day 18). From day 24 onwards, serum levels of CAA were considerably higher than serum levels of CCA. On the other hand, urine samples collected at the day of perfusion (at 6 weeks), showed much higher CCA levels than CAA.[60] This difference between serum and urine in the ratio between the two antigens has also been noticed in infected hamsters,[60] as well as in humans (see also chapter 2.1).[25]

It is not clear whether the disagreement between the *in vitro* and *in vivo* data on the ratio between CAA and CCA, is caused by a difference in metabolic activity resulting in a change in antigen production, or is due to clearance mechanisms of the host. In contrast to the findings of Van Dam and colleagues, Van 't Wout *et al.* found considerably higher levels of CCA compared to CAA in sera of mice, 10 weeks after infection with 100 *S. mansoni* cercariae.[69] These conflicting data may be caused by a difference in antigen clearance, either due to the genetic variation of the mouse strains used, or by the 10 times higher number of cercariae used in the study of Van Dam *et al.*.[60] The latter explanation would suggest a differential clearance of the two antigens depending on the intensity of infection.

Other studies indicated a less important role of antibody-mediated clearance mechanisms. Surgical transfer of male adult *S. mansoni* worms to naive mice showed serum CAA levels to be approximately 10 times higher than CCA during the first week after transfer, when specific antibodies were not yet formed. During the remaining period of the experiment, up to three weeks after transfer, CAA levels continued to be higher than CCA, although only 3 to 4 times (Van Lieshout *et al.*, unpublished data). Another study showed that the transfer of only male worms resulted in substantially different levels and kinetics of CAA, compared to the transfer of worm pairs.[70] Although this could partly be explained by the major role of female worms in the CAA production, other factors also seem to determine antigen production, e.g. the general "well-being" of the parasite.

Studying kinetics of CAA levels in groups of mice infected with five different *Schistosoma* species, Agnew *et al.* found serum levels of CAA to be proportional to worm numbers for all species, even when mice carried worm burdens that were very large relative to host size.[71] In addition, the relation between CAA level and worm burden was not affected in T-cell deprived mice, suggesting that immune-mediated clearance may not be an important factor.[71]

In conclusion, although the levels of CAA and CCA in serum and urine are highly correlated with each other (and with the number of worms present), the

production and clearance of these two antigens appears to be regulated somewhat differently. This has no large implications for the diagnostic application of circulating antigen determination, where other characteristics like the sensitivity and specificity of the assays, as well as the kinetics following successful treatment, are far more important. However, for the epidemiological application of CAA and CCA detection, it is important to know whether the levels of these antigens can truly be interpreted as an alternative for the assessment of worm numbers.

Determination of actual worm burden

Several studies have been published on the relationship between worm burden and antigen concentrations in the serum of experimentally infected animals. Most of them demonstrated a significant correlation between these two parameters,[63,71-78], although some could not find such a relationship.[60,69,79,80] At least in part, this may be explained by improvement over the years of the assays detecting these antigens as well as the sample pre-treatment procedure (needed to dissociate antigen-containing IC and to neutralize interfering proteins). In addition, it seems to be crucial whether one same cercarial infection dose has been used for all animals in the experiment, or whether a range of cercarial doses has been used. A single dose of cercariae results in a narrow range in worm burden of about 2 to 3 times only, while different doses would allow much larger ranges.[60,69,71]

For the epidemiological application of CAA and CCA determination, e.g. to compare different foci, more studies should be performed on antigen production of different parasite strains. Different species have already been compared, but gave conflicting results. One study showed similar CAA levels per worm in *S. mansoni* and *S. japonicum* infected mice, while these levels were approximately 5 times lower in *S. haematobium* infected animals.[71] Another study demonstrated much higher CAA levels in *S. japonicum* infected mice compared to *S. mansoni*, while serum CCA levels were much higher in the latter.[69]

Taking all the different mechanisms influencing production and clearance into account, it can be concluded that, for the time being, CAA and CCA levels can only be used in epidemiological studies as an indication of worm burden at the population level, but are not applicable for actually "counting" the number of worms in individual cases. On the other hand, this shortcoming is even more true when counting parasite eggs.

In conclusion, although quantification of CAA and CCA has shown to have some limitations, our data demonstrated that the demonstration of these antigens provides a realistic alternative to parasitological examination for the diagnosis of an active *Schistosoma* infection and for the assessment of cure. The epidemiological applications of CAA and CCA determination seem to be promising, although some aspects of production and clearance need to be further studied before firm conclusions can be drawn on the kinetics of worm burdens in human populations.

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Summary

The studies described in this thesis have been performed within the scope of ongoing research to improve the diagnosis of human schistosomiasis by the detection of circulating antigens. Several antigens have been described for this purpose; this thesis focuses on two of them which have been most extensively studied: circulating anodic antigen (CAA) and circulating cathodic antigen (CCA). Both CAA and CCA are glycoconjugates originating from the gut of the adult worm.

The main objectives of this thesis were (i) to further evaluate the demonstration of CAA and CCA in serum and urine for the diagnosis of an active *Schistosoma* infection in humans, (ii) to further investigate the use of these antigen-assays for the assessment of cure following chemotherapy, and (iii) to use the quantification of CAA and CCA for more epidemiologically-oriented studies, i.e. for the assessment of worm burdens to study the dynamics of parasite populations in endemic areas.

A general introduction to human schistosomiasis is given in **chapter 1**, including the biology and life cycle of the parasite and the pathology, transmission and treatment of the disease. The most common procedures for the diagnosis of a *Schistosoma* infection are discussed in more detail. The experimental work is described in **chapter 2**.

To analyze which circulating antigen assay or combination of assays is most sensitive for the diagnosis of a *Schistosoma* infection, serum and urine levels of CAA and CCA were compared in one and the same group of Egyptian schistosomiasis patients (**chapter 2.1**). The number of positive cases ranged from 82 to 88% for the individual assays, while this sensitivity could be increased to 91-98% by combining the test results in a parallel manner, i.e. to consider an individual to be positive when showing a positive titre in at least one of the assays. This procedure was possible because of the high specificity of the monoclonal antibody-based ELISA's used for the detection of CAA and CCA.

In **chapter 2.2**, an ELISA is described for the combined measurement of CAA and CCA. For this method, a mixture of an anti-CAA and anti-CCA monoclonal antibody was used, both as coating and as fluorescein-labelled detecting antibodies in a FITC-anti-FITC system. The lower detection limit of this assay was in the same order of magnitude as the individual CAA and CCA assays. However, when evaluating serum samples from *S. mansoni* infected individuals from Burundi and Zaire, it was shown that combined testing for the two antigens in the same serum sample resulted in a lower sensitivity than testing for the individual antigens and combining these results in a parallel manner. This was most apparent in individuals with low to moderate intensity of infection.

The kinetics of CAA in serum and urine before and after treatment with praziquantel is reported in **chapter 2.3**. For this purpose, part of the individuals described in chapter 2.1 were treated with a comparatively high dose of praziquantel (3 times 20 mg/kg, given in one day) and followed for another six weeks. During this period, all individuals were hospitalized, so no reinfection could occur. CAA titers in serum and urine decreased significantly one week after therapy. Thereafter, the profile of CAA level in urine continued to show a parallel but delayed decline compared to that in serum.

In **chapter 2.4**, the kinetics of CCA in serum and urine is reported for the same population as described in chapter 2.3, also before and after treatment with praziquantel. Overall, the decline of CCA levels showed a profile similar to that of serum CAA, i.e. CCA levels dropped significantly within one week and most individuals were found to be negative within 3 to 6 weeks after the chemotherapy. In addition, no circadian variability was found for the urine levels of CAA and CCA, before treatment (chapters 2.3 and 2.4).

In **chapter 2.5**, the use of circulating antigens was evaluated as a tool to monitor the efficacy of different treatment regimens. CAA and CCA levels in serum and urine of Egyptian schistosomiasis patients (described in chapter 2.1) were retrospectively analyzed, following chemotherapy with either 40 or 60 mg/kg praziquantel. Before treatment, all patients were positive for at least one of the antigen assays and no differences were found between the two groups. Three to six weeks after chemotherapy, significantly more cases were found to be negative in the group treated with 60 mg/kg of praziquantel. These results were confirmed by parasitological data.

The applicability of CAA and CCA determination for the diagnosis of schistosomiasis in low endemic areas is described in **chapter 2.6**. Serum and urine samples were collected from a village in Surinam with low prevalence and intensity of *S. mansoni* and tested for CAA and CCA levels. The detection of CAA in serum, CCA in urine, as well as the parasitological examination, showed the highest sensitivity, with 23-29% of the screened individuals found to be positive. However, each individual diagnostic procedure missed a substantial part of the infected cases. From this study it was concluded that for the diagnosis of schistosomiasis in low endemic areas, circulating antigen detection should be considered as a complementary tool rather than a substitute for parasitological examination. Moreover, the diagnostic performance of antibody measurement was found to be surprisingly poor in this population.

The demonstration of CAA and CCA in serum and urine of individuals originating from a non-endemic area and with acute schistosomiasis after exposure on a known date is reported in **chapter 2.7**. The sensitivity of antigen determination was found to be similar to intensive parasitological screening, but lower than the detection of specific antibodies. CAA and/or CCA could be demonstrated in serum in 85% of the cases, starting from 5 or 6 weeks after the onset of infection, respectively. However, serum antigen levels were generally very low, while urines were all found to be negative.

For the detection of CAA and CCA in serum and urine, pretreatment of samples with TCA is a standard procedure. In **chapter 2.8**, a more simple and rapid technique is evaluated, i.e. 30 minutes of heat incubation (70°C) in an alkaline buffer. This technique showed similar sensitivities and specificities for CAA and CCA in serum, and CCA in urine. However, for the determination of CAA in urine, the new technique was not only far more simple to perform, but also resulted in a notable increase of the sensitivity.

Several experimental studies have shown a good correlation between worm loads and the serum levels of CAA and CCA. The use of these antigens for the assessment of worm burdens in humans is reported in **chapter 2.9**. Serum levels of CAA and CCA were determined in a comparatively large population from an endemic area in Zaire with intense *S. mansoni* transmission, and analyzed in relation to the age of the host and the parasite egg counts. Antigen levels showed an age-related pattern similar to egg counts, i.e. a peak in intensity during adolescence. Although it can not be excluded that other mechanisms may regulate the production and/or clearance of CAA and CCA, these results indicate a genuine reduction of worm loads rather than a reduced fecundity with

age of the host, as suggested by others. Apart from that, the relation between CAA and egg counts was found to be nonlinear, which would be consistent with the phenomenon of crowding, i.e. a reduced egg production in hosts with high worm loads.

In **chapter 2.10**, the serum antigen levels from the Zaire population described in chapter 2.9, are compared with serum CAA and CCA levels of a population living in an area in Senegal. In this area prevalences and intensities of *S. mansoni* infections are also very high, but the transmission started only recently. While parasite egg counts were almost similar in the two populations, with a comparable age related distribution, serum levels of both CAA and CCA were approximately twice as high in the chronically exposed community (Zaire) in comparison to the recently exposed community (Senegal). These findings suggest comparatively lower worm burdens, and therefore higher worm fecundity, in the recently exposed community.

This thesis concludes with a general discussion in **chapter 3.** In this chapter, the determination of CAA and CCA is extensively compared with the demonstration of parasite eggs, with an emphasis on the shortcomings of both diagnostic procedures. Special attention is paid to the possible mechanisms involved in the production and clearance of CAA and CCA.

Samenvatting

Het in dit proefschrift beschreven onderzoek vond plaats in het kader van een al langer lopend onderzoek dat is gericht op het verbeteren van de diagnostiek van humane schistosomiasis door middel van het aantonen van circulerende antigenen. Verscheidene van deze antigenen zijn voor dit doel reeds beschreven. Dit proefschrift richt zich op het aantonen in serum en urine van twee van deze antigenen die relatief het meest uitgebreid zijn bestudeerd en gekarakteriseerd, te weten *circulating anodic antigen* (CAA) en *circulating cathodic antigen* (CCA). Beide antigenen worden gekenmerkt door koolhydraat-structuren en zijn afkomstig uit de darm van de volwassen parasiet.

De voornaamste doelstellingen van het in dit proefschrift beschreven onderzoek waren (i) een verdere evaluatie van de mogelijkheden om CAA en CCA in serum en urine aan te tonen voor het diagnostiseren van een actieve *Schistosoma* infectie bij de mens, (ii) voortgezet onderzoek naar het gebruik van testen die deze antigenen aantonen om te kunnen bepalen of genezing heeft plaatsgevonden na behandeling van de parasitaire infectie, en (iii) het bestuderen van de toepassing van CAA en CCA bepalingen voor meer epidemiologisch gerichte studies, met name voor het schatten van wormlasten om de dynamiek van de parasiet populaties in endemische gebieden te onderzoeken.

Hoofdstuk 1 bevat een algemene inleiding over schistosomiasis. Daarbij wordt een korte beschrijving gegeven van de biologie en levenscyclus van de parasiet en van de pathologie, overdracht en behandeling van de ziekte. Dit hoofdstuk bespreekt tevens de meest gebruikelijke methoden voor het diagnostiseren van een *Schistosoma* infectie.

Hoofdstuk 2 beschrijft het experimentele werk. Om te analyseren welke van de circulerend antigeen-testen of combinatie van testen het meest gevoelig is voor het aantonen van een *Schistosoma* infectie, wordt in **hoofdstuk 2.1** het niveau van CAA en CCA in serum en urine binnen dezelfde groep van Egyptische schistosomiasis patiënten vergeleken. Het aantal positieve individuen varieerde per antigeen-test van 82 tot 88%. Deze gevoeligheid kon worden ver-

hoogd tot 91-98% door middel van het op parallelle wijze combineren van de afzonderlijke testresultaten, d.w.z. een individu wordt als positief beschouwd wanneer op zijn minst één van de antigeen-testen een positieve uitslag vertoont. Deze procedure was mogelijk door de hoge specificiteit van de ELISA-testen voor het aantonen van CAA en CCA, welke gebaseerd zijn op het gebruik van monoclonale antilichamen.

Hoofdstuk 2.2 beschrijft een ELISA-test voor het gelijktijdig aantonen van CAA en CCA. Hiervoor is gebruik gemaakt van een mengsel van een anti-CAA en een anti-CCA monoclonaal antilichaam, zowel voor de coating als voor het aantonen van de antigenen. Voor dit laatste werden de monoclonale antilichamen geconjugeerd met een fluorochroom en toegepast in een FITC-anti-FITC systeem. De detectielimiet van deze test was in dezelfde orde van grootte als van de individuele CAA en CCA ELISA's. Bij het beoordelen van serum monsters van personen uit Burundi en Zaïre met een *S. mansoni* infectie werd echter duidelijk dat het gelijktijdig aantonen van CAA en CCA resulteerde in een lager aantal positieve gevallen dan het op parallelle wijze combineren van de individuele test-uitslagen. Dit gold vooral voor individuen met een minder hoge intensiteit van de infectie.

In **hoofdstuk 2.3** wordt de kinetiek bestudeerd van CAA in serum en urine, voor en na behandeling met het geneesmiddel praziquantel. Voor dit doel werd een deel van de in hoofdstuk 2.1 beschreven patiëntengroep gedurende zes weken gevolgd na behandeling met een relatief hoge dosis praziquantel (3 keer 20 mg/kg, verspreid over één dag). Tijdens deze periode verbleven de patiënten in een ziekenhuis, zodat geen herinfectie kon optreden. Een week na behandeling was de afname van CAA in serum en urine significant. Daarna zakte het niveau van CAA in urine langzamer dan het niveau in serum.

Hoofdstuk 2.4 beschrijft de kinetiek van CCA in serum en urine voor en na behandeling met praziquantel voor dezelfde patiëntengroep als beschreven in hoofdstuk 2.3. In het algemeen vertoonde de afname van CCA in serum en urine een patroon vergelijkbaar met dat van CAA in serum, d.w.z. dat het niveau significant lager was één week na de therapie en bij een meerderheid van de individuen na drie tot zes weken negatief. Tevens vertoonden de concentraties van CAA en CCA in urine geen circadisch ritme (hoofdstukken 2.3 en 2.4).

In **hoofdstuk 2.5** wordt het gebruik van circulerend antigeen-testen geëvalueerd om verschillende behandelingstherapieën te kunnen vergelijken. Voor dit doel werd de kinetiek van CAA en CCA in serum en urine van een groep Egyp-

tische schistosomiasis patiënten (beschreven in hoofdstuk 2.1) retrospectief geanalyseerd gedurende zes weken na therapie. Een deel van deze patiënten was behandeld met 60 mg/kg praziquantel, een ander deel met de standaard dosis van 40 mg/kg. Voor behandeling was de uitslag van op zijn minst één van de antigeen-testen voor alle patiënten positief en vertoonden de twee behandelingsgroepen geen verschil in circulerend antigeen-spiegels. Drie tot zes weken na de therapie waren er in de groep die behandeld was met 60 mg/kg praziquantel significant meer patiënten negatief voor CAA en CCA. Deze resultaten werden bevestigd door het parasitologische onderzoek.

Hoofdstuk 2.6 beschrijft de toepassing van het aantonen van CAA en CCA voor het diagnostiseren van schistosomiasis in een laag endemisch gebied. Serum- en urinemonsters werden verzameld in een dorp in Suriname met lage prevalentie en intensiteit van *S. mansoni*, en daarna getest op CAA en CCA. De testen voor het aantonen van CAA in serum, CCA in urine en het parasitologische onderzoek, bleken daarbij het gevoeligst met een positieve uitslag variërend van 23 tot 29%. Elke afzonderlijke test miste echter een aanzienlijk deel van de geïnfecteerde gevallen. Uit dit onderzoek kon worden geconcludeerd dat, in het geval van het diagnostiseren van schistosomiasis in laag endemische gebieden, het aantonen van circulerende antigenen meer beschouwd moet worden als een aanvulling, dan als een vervanging van het parasitologische onderzoek. Daarnaast bleek bij deze studiepopulatie de diagnostische waarde van het aantonen van specifieke antilichamen opvallend laag.

Hoofdstuk 2.7 beschrijft het aantonen van CAA en CCA in serum en urine bij een groep personen, afkomstig uit een niet-endemische gebied, met een acute vorm van schistosomiasis na een eenmalige blootstelling. Het aantonen van circulerende antigenen vertoonde eenzelfde gevoeligheid als dat van een zeer uitgebreid parasitologisch onderzoek, maar een lagere gevoeligheid dan het aantonen van specifieke antilichamen. In serum kon CAA en/of CCA worden aangetoond in 85% van de individuen, vanaf respectievelijk 5 en 6 weken na de aanvang van de infectie. Antigeen-spiegels waren echter erg laag en alle urine monsters waren negatief.

Voor het aantonen van CAA en CCA in serum en urine worden monsters standaard voorbehandeld met TCA. **Hoofdstuk 2.8** beschrijft een simpeler en snellere voorbehandelingsmethode, namelijk een 30 minuten durende verhitting in een alkalische buffer. Deze methode resulteerde in een gelijke gevoeligheid en specificiteit voor het aantonen van CAA en CCA in serum en CCA in urine. Voor het aantonen van CAA in urine bleek deze methode bij de praktische uitvoering

niet alleen vele malen eenvoudiger, maar ook te leiden tot een aanzienlijke toename in gevoeligheid.

Verscheidene proefdierstudies hebben laten zien dat de concentratie van CAA en CCA in de circulatie van de gastheer een goede correlatie vertoont met het aantal aanwezige parasieten. **Hoofdstuk 2.9** beschrijft het gebruik van deze antigenen ter indicatie van de wormlast bij mensen. Voor dit doel werden CAA- en CCA-spiegels bepaald in serum monsters van een relatief grote studiepopulatie afkomstig uit een gebied in Zaïre met een zeer hoog niveau van *S. mansoni* transmissie. Antigeen-spiegels werden geanalyseerd in relatie tot de leeftijd van de gastheer en het aantal eieren in de ontlasting. De leeftijdsgebonden distributie van antigeen-concentraties kwam overeen met die van de eitellingen, d.w.z. beide vertoonden een piek gedurende de puberteit. Hoewel niet kan worden uitgesloten dat andere factoren de productie en/of klaring (opruiming) van CAA en CCA kunnen beïnvloeden, geven deze resultaten aan dat de karakteristieke daling in eitellingen bij de volwassen gastheer wordt veroorzaakt door een werkelijke afname in het aantal wormen en niet door een afname van de ei-productie van de parasiet, zoals door andere onderzoekers is gesuggereerd. Tevens werd een niet-lineaire relatie gevonden tussen de serumconcentratie van CAA en het aantal eieren in de ontlasting. Dit is in overeenstemming met het fenomeen van *crowding*, d.w.z. een afname in ei-productie bij infecties met zeer hoge worm aantallen.

In **hoofdstuk 2.10** worden de serum CAA- en CCA-spiegels beschreven in hoofdstuk 2.9 vergeleken met die van een studiepopulatie afkomstig uit Senegal. Het betreft een gebied met een recente *S. mansoni* transmissie, maar met eveneens een zeer hoge prevalentie en intensiteit van de infectie. De twee studiepopulaties vertoonden eenzelfde hoeveelheid eieren in de ontlasting, met tevens een vergelijkbare leeftijdsgebonden distributie. De concentraties van CAA en CCA waren daarentegen significant hoger in de populatie welke sinds enkele decennia aan de ziekte had blootgestaan (Zaïre). Deze resultaten suggereren zowel het voorkomen van relatief lagere aantallen wormen, als van een hogere ei-productie in de populatie met recentere infecties (Senegal).

Dit proefschrift wordt in **hoofdstuk 3** afgesloten met een algemene discussie. Daarin wordt een uitgebreide vergelijking gemaakt tussen het bepalen van CAA en CCA en het detecteren van eieren van de parasiet, met de nadruk op de onvolkomenheden van beide diagnostische procedures. Speciaal wordt aandacht besteed aan de mechanismen die een rol zouden kunnen spelen bij de productie en klaring (opruiming) van CAA en CCA.

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Curriculum vitae

Lisette (Elisabeth Antoinette) van Lieshout was born on February 3, 1964 in Amsterdam (The Netherlands). In 1982 she finished the Amsterdam Montessori Lyceum (VWO). After a year of travelling and working in several European countries, she started her study Biology at the University of Amsterdam (UvA) in 1983, later specializing in Medical Biology. For her main subject, she performed one year of research on cellular immunology in the Department of Histology and Cell-biology (UvA). After finishing the obligatory exams in 1987, she carried out an additional one year main subject in the field of parasitology, performed in three different institutes: the Department of Parasitology of the University of Leiden (RUL), the Theodor Bilharz Research Institute in Cairo and the Urology and Nephrology Center of the University of Mansoura, both located in Egypt. In 1988, she obtained her Masters Degree (doctoraal examen), after which she started working as a guest investigator at the Schistosomiasis Department (Division of Basic Research) of the Naval American Medical Research Unit-3 (NAMRU-3) in Cairo, Egypt. In 1991, she returned to the Netherlands, and joined the Department of Parasitology (RUL) for a period of four years (AIO-position), to continue the research described in this thesis on the immunodiagnosis of schistosomiasis by the detection of circulating antigens. In 1995, she was appointed in the same department as a post-doc (funded by NWO-WOTRO), to study the immuno-epidemiological aspects of a recent outbreak of schistosomiasis mansoni in Northern Senegal.