Candidate gene family-based and case-control studies of susceptibility to high *Schistosoma mansoni* worm burden in African children: a protocol [version 1; peer review: 1 approved]

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**Abstract**

**Background:** Approximately 25% of the risk of *Schistosoma mansoni* is associated with host genetic variation. We will test 24 candidate genes, mainly in the Th2 and Th17 pathways, for association with *S. mansoni* infection intensity in four African countries, using family based and case-control approaches.

**Methods:** Children aged 5-15 years will be recruited in *S. mansoni* endemic areas of Ivory Coast, Cameroon, Uganda and the Democratic Republic of Congo (DRC). We will use family based (study 1) and case-control (study 2) designs. Study 1 will take place in Ivory Coast, Cameroon, Uganda and the DRC. We aim to recruit 100 high worm burden families from each country except Uganda, where a previous study recruited at least 40 families. For phenotyping, cases will be defined as the 20% of children in each community with heaviest worm burdens as measured by the circulating cathodic antigen (CCA) assay. Study 2 will take place in Uganda. We will recruit 500 children in a highly endemic community. For phenotyping, cases will be defined as
the 20% of children with heaviest worm burdens as measured by the CAA assay, while controls will be the 20% of infected children with the lightest worm burdens. Deoxyribonucleic acid (DNA) will be genotyped on the Illumina H3Africa SNP (single nucleotide polymorphisms) chip and genotypes will be converted to sets of haplotypes that span the gene region for analysis. We have selected 24 genes for genotyping that are mainly in the Th2 and Th17 pathways and that have variants that have been demonstrated to be or could be associated with *Schistosoma* infection intensity.

**Analysis:** In the family-based design, we will identify SNP haplotypes disproportionately transmitted to children with high worm burden. Case-control analysis will detect overrepresentation of haplotypes in extreme phenotypes with correction for relatedness by using whole genome principal components.

**Keywords**
S. mansoni, infection intensity, family based linkage, haplotypes, candidate genes
**Abbreviations**

BMI: Body mass index  
CAA: Circulating anodic antigen  
CCA: Circulating cathodic antigen  
DRC: Democratic Republic of Congo  
FBAT: Family based association test  
H3Africa: Human Heredity and Health in Africa research consortium  
KK: Kato Katz  
LaVIISWA: Lake Victoria Island intervention study on worms and allergy  
LOD: Logarithm of odds  
MAF: Minor allele frequency  
MDA: Mass drug administration  
MUAC: Mid Upper arm circumference  
PCR: Polymerase chain reaction  
POC-CCA: Point of care-circulating cathodic antigen test  
PZQ: Praziquantel  
QTL: Quantitative trait locus/loci  
RDT: Rapid detection test  
REC: Research ethics committee  
SM1: Schistosoma mansoni susceptibility locus 1  
SM2: Schistosoma mansoni susceptibility locus 2  
SRR: Sibling Recurrence Risk  
TDT: Transmission disequilibrium test  
Th1: T helper cell 1 cells/pathway of immunity  
Th2: T helper cell 2 cells/pathway of immunity  
Th17: T helper 17 cells/pathway of immunity  
UCP-LF: Upconverting phosphor lateral flow  
UNCST: Uganda National Council of Science and Technology  
UVRI: Uganda Virus Research Institute  
WHO: World Health Organization

**Introduction**

Schistosomiasis is a major parasitic disease with prevalence estimates ranging from about 140 million to more than 250 million people worldwide, while nearly one billion are at risk (Colley et al., 2014; LoVerde, 2019; McManus et al., 2018). In 2016, at least 206.5 million people required preventive treatment and more than 40 million people were treated for the parasite according to the WHO. Currently, 90% of all cases, and most severe cases, are in sub-Saharan Africa (Beltrame et al., 2017; Mayaka, 2001). There are 8 to 10 million new cases reported each year worldwide, with 800,000 deaths per year globally and between 200,000 and 400,000 deaths per year in the sub-Saharan African region (Saotoing et al., 2014; WHO, 2013).

Schistosomiasis is linked to poverty, affects the socio-economic development of populations (Adenowo et al., 2015) and represents a significant burden for developing countries, particularly African countries (Mnkugwe et al., 2020). The major species prevalent in African countries are *S. mansoni*, which causes intestinal schistosomiasis and *S. haematobium*, which causes urinary schistosomiasis (Colley et al., 2014). School age children have the highest worm burdens (Mnkugwe et al., 2020; Saotoing et al., 2014).

Mass drug administration (MDA) in school children, snail control programs and improved sanitation are all important to the control of schistosomiasis, but each approach is held back by serious technical difficulties and inadequate resources.

MDA has been the major strategy employed and all children in high prevalence areas are treated irrespective of whether they are infected or not. The frequency of MDA (every six months or every one or two years) is dependent on the prevalence of schistosomiasis in a region (Montresor & World Health Organization, 2011). MDA could be targeted to the most susceptible children; however, there is an incomplete understanding of host genetics of susceptibility, requiring more studies to guide this strategy.

We have reviewed genetic studies of the human response to schistosome infection and found the associations have been reported between schistosome worm burdens and 24 single nucleotide polymorphisms (SNP) in 11 candidate genes (Mewamba et al., 2021). Mapping studies have shown that there are at least three quantitative trait loci (QTL) regions of the genome that regulate the response to infection, however the genes that are responsible for these observations have not been identified (Mewamba et al., 2021). Although candidate gene studies are a powerful strategy for discovering associations between SNP and phenotype, the majority of such observations have not been replicated when studies have been repeated in different populations (Hirschhorn et al., 2002). Whilst two of the 24 SNP associated with schistosomiasis have replicated in additional populations, four have failed to replicate when tested and no attempt has been made to replicate the remaining 18 (Mewamba et al., 2021). We will undertake replication studies for all eleven of these genes in populations from Ivory Coast, Cameroon, Uganda and the Democratic Republic of Congo that are endemic for *S. mansoni* schistosomiasis and that have not been previously included in association studies (Table 1). We will test additional genes that are in QTL and that are known to be involved in the response to schistosome infection but...
We will also test genes in the Th17 pathway that have not been included in genetic studies of schistosomiasis. We will genotype using the H3Africa Illumina Omni SNP chip that contains approximately 2.5m SNP. This will enable us to construct haplotypes across whole gene regions for association tests, rather than relying on individual SNP.

The study will sample intensively within a small number of communities in four countries (Ivory Coast, Cameroon, Uganda and the Democratic Republic of Congo) where schistosomiasis prevalence is high. Under these conditions case control studies within communities are at risk of being confounded by cryptic relatedness which can cause type 1 errors (false positives). The large number of candidate gene case control studies that have not been replicated may be partially a consequence of population structure (Mewamba et al., 2021). We will use two strategies to control for population structure: 1) family-based study designs, which are immune to errors caused by population sub-structure (Laird & Lange, 2009); 2) a case-control

<table>
<thead>
<tr>
<th>Gene names</th>
<th>Reference (Novel)</th>
<th>Justifications</th>
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<tbody>
<tr>
<td>IFNG</td>
<td>(Gatlin et al., 2009)</td>
<td>SM2 Chr6 Region</td>
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<tr>
<td>IL10</td>
<td>(Adedoya et al., 2018; Grant et al., 2011)</td>
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<tr>
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<td>(Gatlin et al., 2009)(Grant et al., 2012; Isnard et al., 2011; Kouriba et al., 2005)</td>
<td>SM1 Chr5 Region</td>
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<td>IL4</td>
<td>(Adedokun et al., 2018) (Gatlin et al., 2009)</td>
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<tr>
<td>IL5</td>
<td>(Ellis et al., 2007)</td>
<td>SM1 Chr5 Region</td>
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<tr>
<td>STAT6</td>
<td>(Adedokun et al., 2018; He et al., 2008)</td>
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<td>CTLA4</td>
<td>(Idris et al., 2012)</td>
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<td>FCN2</td>
<td>(Ouf et al., 2012)</td>
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<td>COLEC11</td>
<td>(Antony et al., 2015)</td>
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<td>ABO</td>
<td>(Tiongco et al., 2018)</td>
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<td>IL12B</td>
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<td>SM1 chr 5 QTL, Th1 and Th17 pathways</td>
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<td>Novel (0)</td>
<td>Chr 6p21-q21 QTL, implicated in granuloma formation</td>
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<tr>
<td>IL17B</td>
<td>Novel (0)</td>
<td>SM1 chr 5 QTL, implicated in granuloma formation</td>
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<td>IL21</td>
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<td>Mediate the Th17 Cell amplification</td>
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<td>IL23A</td>
<td>Novel (1, Coeliac)</td>
<td>Stabilize Th17 Cell production</td>
</tr>
<tr>
<td>RNASE3 (ECP)</td>
<td>(Eriksson et al., 2007)</td>
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have not been previously included in genetic association studies. We will also test genes in the Th17 pathway that have not been included in genetic studies of schistosomiasis. We will genotype using the H3Africa Illumina Omni SNP chip that contains approximately 2.5m SNP. This will enable us to construct haplotypes across whole gene regions for association tests, rather than relying on individual SNP.

The study will sample intensively within a small number of communities in four countries (Ivory Coast, Cameroon, Uganda and the Democratic Republic of Congo) where schistosomiasis prevalence is high. Under these conditions case control studies within communities are at risk of being confounded by cryptic relatedness which can cause type 1 errors (false positives). The large number of candidate gene case control studies that have not been replicated may be partially a consequence of population structure (Mewamba et al., 2021). We will use two strategies to control for population structure: 1) family-based study designs, which are immune to errors caused by population sub-structure (Laird & Lange, 2009); 2) a case-control
design with correction for population structure using principal components calculated from genome wide SNP data.

Estimating intensity of infection is also a problem for schistosomiasis studies. The Kato-Katz method of counting eggs in stool samples is known to lack sensitivity and this is compounded by day to day variation in egg excretions and uneven distribution of eggs across an individual stool (Kongs et al., 2001).

The proposed study aims to improve on previous studies by using:

1) A more reproducible phenotyping method (CCA or CAA)
2) A more systematic selection of candidate genes (Table 1)
3) A study design that is less sensitive to population structure

The specific study aims are as follows:

1) Test candidate genes listed in Table 1 for association with intensity of *S. mansoni* infection
2) Test if previously published associations are replicated in other populations

The hypotheses we will test are:

i) Variation in Th2 and Th17 pathway genes contribute to variation in *S. mansoni* worm burden
ii) Genes in the Th17 pathway contribute to the association with schistosomiasis at three QTL
iii) Associations found with genes in the SM1 and SM2 QTL are replicable across multiple populations

**Protocol**

**Ethical approval and consent to participate**

The study will be carried out in Cameroon, Uganda, DRC and Ivory Coast. In Cameroon the study was approved by the National Ethics Committee for research on human health of the Ministry of Public Health of Cameroon with the reference number N°2019/02/1144/CE/CNERSH/SP. The review board of the Molecular Parasitology and Entomology Subunit of the Department of Biochemistry of the Faculty of Science of the University of Dschang gave its approval.

In Uganda, the approval was from the Uganda National Council for Science and Technology (UNCST) number: HS 1183 and the Uganda Virus Research Institute Research Ethics Committee: approval of amendment to perform genetic study on human susceptibility to schistosomiasis Ref: GC/127/18/09/317

In DRC, the ethics review number is COMETH/TKK/PRES/004/2018 approved by the Clinic Ngaliema Ethical Committee of the Health Ministry.

In Ivory Coast, the study was approved by the National Ethics Committee for Life Sciences and Health (cnesvs); approval number N/Ref: 040-19/MSHP/CNESVS-kp.

**Study design**

Two study designs will be employed in different countries, described in detail below.

**Study 1: Family based linkage study; to be conducted in the Democratic Republic of Congo, Ivory Coast, Cameroon and the Uganda Lake Victoria islands.**

**Study 2: Case control study; to be conducted in the Lake Albert Region of Uganda.**

**Study 1: Family based linkage and association study**

**Study sites**

Uganda: previous collections by the Lake Victoria Island Intervention Study on Worms and Allergy (LaVIISWA), conducted on the Lake Victoria islands of Koome.

Ivory Coast: Bloquin and Guiglo (West)

Cameroon: Makenene and Adamawa regions

DRC: Kongo Central (Kisantu and Kimpese)

**Participant recruitment**

Consent will be obtained in two stages.

1) Written collective consent for screening for schistosome worm burden will be provided by school directors or health authorities.
2) Written informed consent for children who meet inclusion criteria to participate in the study will be requested from parents or guardians in the home after children have been screened for intensity of infection at school.

The informed consent forms and case report forms are provided as part of extended data in Harvard Dataverse (Noyes, 2021a).

Community entry procedures differ in each country as mandated by the respective ethical review committees. In DRC administrative authorities, school inspectors, school directors and teachers were involved, whereas in Ivory Coast administrative authorities, Regional Health Director, Departmental Health Director school inspectors, school directors and teachers were involved. In Cameroon, the following were notified about the study; Ministry of Health and Ministry for Schools for Region, school inspectors, school directors and teachers were involved. In Uganda, the ministry of health and village health teams were involved in community entry.

Subsequently collective consent will be obtained from district health authorities or school directors and the date of the
screening will be planned. The parents or guardians of children at schools will be informed by school directors of schistosomiasis screening at the school on a given date and invited to let their children participate. In Cameroon, DRC and Ivory Coast, schools were chosen that were in high S. mansoni endemic areas. The number of schools is not defined at the beginning as it is dependent on the number of high worm burden families that can be recruited in the school. We will continue to sample schools until we have recruited our target number of children, which is at least 100 families per country.

After screening children for intensity of infection, parents or guardians of children who meet inclusion criteria will be visited and invited to provide informed consent for themselves and their participating children. Children older than 10 years will be invited to provide assent. Each participant will be free to choose whether to join the study after the objectives, procedures, and potential risks and benefits of the study have been clearly explained to them using lay terms.

**Screening procedures.** Schistosomiasis screening will be performed at schools by a team from TrypanoGEN+ together with the National Control Programs team. Children who agree to participate in the study will be tested for S. mansoni infection by detecting circulating cathodic antigen in their urine and Schistosoma mansoni eggs in their stools, using the Point Of Care Circulating Cathodic Antigen (POC-CCA) test (van Dam et al., 2004) and the Kato Katz (KK) technique respectively (World Health Organisation, 1994). Urine and stool will be collected in plastic collection tubes and bags. In each country, some samples may be kept in the institution which will conduct the research, i.e. University of Dschang (Cameroon samples), INRB (DRC) and University Jean Lorougnon Guédé University (Ivory Coast). All children will be treated with Praziquantel by the national Ministry of Health Mass Drug Administration teams according to the recommendation of the World Health Organization (World Health Organisation, 2006).

**Inclusion criteria**

1. Age between 5-15 years
2. Intensity of infection in the top 20% of positive children in their community
3. Have at least one parent available for inclusion in the study

**Exclusion criteria**

1. No parent available
2. Children positive for S. haematobium by urine filtration test or PCR
3. Treatment with praziquantel for schistosomiasis in the last six months

**Procedures for the top 20% index children in families.** The families of the children bearing high worm burden infections will be visited in their homes to meet their parent or guardian and inform them about the objectives and goals of the study. The head of the families that meet the inclusion and not the exclusion criteria will be invited to give their consent for their family to participate to the study. Adult participants will also be invited to give their consent and the index child and siblings will give their assent if over 10 years. Children, who are encountered in the home and have not been screened at school and meet the enrolment criteria, will be tested for inclusion in the study. Each person who gives consent or assent and meets the enrolment criteria will be asked to provide about 5ml of blood. This will be done in the home and samples will be kept at cold storage for transportation to the relevant laboratory; University of Dschang (Cameroon samples), INRB (DRC) and University Jean Lorougnon Guédé University (Ivory Coast).

**Figure 1** shows a flow-chart of the procedures for this study.

**Timelines.** Timelines for the study are shown in Table 2. Initially, all work was projected to take 3 years. However, collections started in 2019 and may continue until 2023 due to restrictions imposed by the coronavirus disease 2019 (COVID-19) pandemic.

**Diagnosis**

**Kato Katz test**

Each participant will provide a stool sample for the Kato-Katz test (KK). The WHO approved standard KK test will be conducted (Katz et al., 1972).

This technique can be used to distinguish the different helminth species eggs (S. mansoni Ascaris lumbricoides, Trichuris trichiura, hookworm and Taenia spp), which will be recorded separately.

**Circulating cathodic antigen (CCA) test**

The KK thick stool smear is the most common field technique for S. mansoni identification and quantification. This method is 100% specific, however it has poor sensitivity for detecting low intensity infections (Colley et al., 2017). More recently the CCA that is produced by adult worms (Nash & Deelder, 1985) and is detectable in blood and urine, has been used as the basis for a lateral flow test that can be used at the point of care (Kittur et al., 2016; WHO, 2012). The advantages of the POC-CCA test include its rapid flow, easy handling under field conditions and minimal practical training requirements for its application (Glinz et al., 2010). Several studies have shown that this test appears to be more sensitive than the KK technique (Deelder et al., 1976; Kittur et al., 2016; Mnkugwe et al., 2020). Nevertheless, the specificity of POC-CCA is influenced by cross-reactivity with other helminth infections and, more importantly, may produce false positives with weak bands especially in areas of moderate and low prevalence and this can lead to overestimation of prevalence (Colley et al., 2017). However in areas of high transmission, such as those where studies 1 and 2 are being undertaken, all trace positives should be treated as positives (Colley et al., 2020). Although the abundance of CCA antigen is believed to be proportional to the number of worms present (van Dam et al., 2004), the POC-CCA is marketed as a qualitative test for S. mansoni.
diagnosis and not for worm quantification. However, three studies have demonstrated that there is a semi-quantitative relationship between band intensity estimated visually and egg count on the Kato-Katz test (Coulibaly et al., 2013; Dawson et al., 2013; Kittur et al., 2016). This has recently been improved with a set of 10 standards reference cassettes with ink jet printed bands of increasing intensity (Casacuberta-Partal et al., 2019). We have recently extended this method by using the ESEQuant LR3reader to read the intensity of the positive band. In field trials ESEQuant readings correlated well with estimates of band intensity using the reference cassettes developed by Casacuberta-Partal et al. (2019) with a Spearman rank correlation.
coefficient = 0.89 (Mewamba et al., 2021). We will therefore use the ESEQuant LR3 reader to objectively quantify band intensity on POC-CCA cassettes in a standardized way at all sites in Study 1 except the samples from Lake Victoria. The samples from the Uganda Lake Victoria site are already archived and worm burdens will be quantified using the more sensitive CAA assay.

Using the RDT-Reader for quantitative POC-CCA
The ESEQuant LR3 Gold reader (part number ESLR11-MB-6401 with a drawer inlay for the POC-CCA cassette part number ESLR05-MA-5167 DIALUNOX GmbH, Germany) will be used to measure reflective signals on POC-CCA cassettes. This gives an objective measure different from the subjective visual results. First, a method is developed on the reader to capture valid signals for the control line and test lines. This method will be deployed on subsequent RDT tests on this reader. A quantitative test will be done on the POC-CCA strip by spotting a drop of urine on the POC-CCA. The sample number will be pre-entered on the reader, and once 20 minutes elapse the cassette will be put into the reader and the method will be initiated to read the cassette. Cassettes that are not read at 20 minutes will be discarded and the test will be repeated. An OD signal indicating the intensity of signal will be shown. The intensity per sample is stored on the reader and can be downloaded to compare signal intensities across various samples.

Circulating anodic antigen
The circulating anodic antigen (CAA) assay is a highly sensitive assay that is used to detect the CAA antigen regurgitated by adult Schistosoma worms and is present in urine, plasma and serum (van Dam et al., 2013). The CAA is much more abundant in plasma than CCA is in urine leading to a more sensitive test.

We will use the schistosome CAA assay that requires 20µl of plasma (SCAA20). Since the POC-CCA assay has only been used as a semi-quantitative test (Coulibaly et al., 2013; Dawson et al., 2013; Kittur et al., 2016) we will validate the CCA test against the CAA test in 100 samples. A previous comparison of the two methods in a low endemicity community in Brazil found only modest correlation (Spearman’s rho = 0.24) between CAA and CCA readings which were classified as negative, trace or positive (Sousa et al., 2019). In that study 36% of samples were positive by CAA, 12% by CCA and 1.6% by Kato-Katz demonstrating the relative sensitivity of the methods. CAA results will be compared against 100 CCA results for study 1 to validate CCA reader signals ability to pick out the top 20% of worm burden. We calculated the power to detect a correlation between CAA and CCA signal intensities using the pwr.r.test function in the R pwr library. 100 samples will have 80% power to detect a correlation (r > 0.28). We will also use the CAA test as the primary measure of worm burden for the Lake Victoria samples.

Human negative plasma will be spiked with a known concentration of CAA and dilutions made. A negative plasma sample that has not been spiked with CAA will be included as a negative control. Plasma samples and standards will be extracted with an equal volume of 4% w/v trichloroacetic acid, vortexed and incubated for 5 minutes. Of the resulting TCA soluble fraction, 20µL will be added to wells coated with UCP particles and anti-mouse monoclonal anti-CAA antibodies hydrated with 100µl of high salt lateral flow buffer. The wells will then be incubated for one hour at 37°C while shaking at 900rpm in a shaking incubator. After one hour, the lateral flow strips will be placed in UCP bound wells and the samples will be allowed to flow. The strips will be dried overnight and analysed using the Labrox Upcon scanner (serial number 2180023). The test line (T) signals will be normalized to the flow control (FC) signals of the individual strips and the results expressed as ratio values i.e. T/FC.

Urine filtration and PCR to exclude S. haematobium
Since S. mansoni CCA can also cross react with S. haematobium CCA, all CCA positive samples should be processed further to exclude S. haematobium co-infected samples. This will leave only the S. mansoni mono-infected samples for analysis. Urine filtration followed by microscopy will be used in the field as a screening test to exclude S. haematobium co-infected samples. All S. haematobium negative samples in the urine filtration will be further tested with S. haematobium specific PCR, a more sensitive test to exclude S. haematobium co-infection. Where urine filtration test is not available, all POC-CCA positive urine samples will directly undergo the PCR test without prior screening with the urine filtration test. The S. haematobium PCR works best on urine concentrates. S. haematobium positive samples will be identified by amplifying the repetitive Dra1 sequence which is specific to S. haematobium using primers identified by Hamburger et al., 2001 (Hamburger et al., 2001).

Genotyping and association testing
Candidate genes
Whilst many genes are known to participate in the response to schistosome infection (Kamdem et al., 2018) there is a more limited set of genes that may have variants that contribute

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<th>Year 2</th>
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to the outcome of infection (Mewamba et al., 2021). A list of genes that will be tested is shown in Table 1. Candidate genes have been selected on three principles: 1) genes that have previously been shown to be associated with schistosomiasis in other studies; 2) genes in the QTL regions that have been reported to be involved in the response to schistosome infection; 3) genes that are involved in the Th_3 pathway. We have previously shown that Th_3 pathway genes are present in multiple quantitative trait loci and may contribute to the effect associated with these loci (Mewamba et al., 2021). Further justification for the genes selected is in the extended data (Noyes, 2021b) and in (Mewamba et al., 2021).

Genotyping
DNA will be extracted from whole blood from index cases and their family members using the Qiagen mini kit (QIAamp DNA minikit catalogue number 51306, Qiagen, Germany) or the Quick-DNATM Miniprep Plus Kit (Catalogue Number: D4069, Zymo research, USA) according to manufacturer’s instructions. Genotyping will be done by Illumina (San Diego) on the Illumina H3Africa Pan African V2 chip (The H3Africa Consortium et al., 2014) which contains approximately 2.5 million SNPs enriched for SNP that are informative in African populations.

Family based analyses
We will use family based analysis methods as reviewed by Ott et al. (Ott et al., 2011). The method applied will depend on the structure of families collected. Here we describe one such method, the transmission disequilibrium test (TDT). The TDT assesses evidence of association between markers and schistosomiasis in the presence of linkage. The test statistic (also known as McNemar’s Test) is chi squared distributed

\[ \chi^2 = \frac{(T - NT)^2}{(T + NT)} \]

where T is the number of times the allele of interest is transmitted from heterozygous parents to affected offspring and NT is the number of times that allele is not transmitted (Spigelman et al., 1993). We will use the family based association test (FBAT V1.4) implementation of the TDT (Laird et al., 2000) to test each haplotype allele in each haplotype block for association with schistosomiasis. If there are sufficient families with more than one affected sib, then we will also use the affected sib-pair test in the same package. We will use the multi-locus transmission disequilibrium test (M-TDT) method (Loucoubar et al., 2017) to search for evidence of additive and epistatic effects of multiple loci.

Meta-analysis
Initially data sets from each country with at least 100 families will be analysed independently to identify loci that are associated with populations in each country. Subsequently the data from all four participating countries will be combined in order to undertake a meta-analysis and identify loci that are of smaller effect than are detectable in local studies and that are also consistently associated with disease in multiple African countries. We will use R version 4 for plotting graphics and supplementary data, FBAT V1.4 will be used for fbat analysis and Plink V1.90 for transmission disequilibrium tests (TDT).

Association analysis with haplotypes
Family based methods for the discovery of linkage and association depend on being able to calculate the probability of the child genotypes given the genotypes of the parents. These calculations are only meaningful if there is more than one allele that the children might inherit from each parent, i.e. the parents need to be heterozygous in order for them to be informative at a particular locus.

Since SNP usually only have two alleles the frequencies of heterozygote parents will generally be low. For example, with the highest possible minor allele frequency (MAF) of 50%, 50% of parents will be heterozygous and only 25% of families will have two heterozygous parents. The frequency of heterozygotes can be increased by using SNP haplotypes that also have more alleles. In order to test the viability of using haplotypes, rather than SNP, for the association analysis we ran a simulation using 1,000 Genomes Project human sequence data (Abecasis et al., 2012) to discover the empirical distribution of haplotype frequencies. Having identified 378 haplotype blocks within the 24 candidate genes we ran a power analysis to discover the proportion of loci where there was adequate power to discover different values of haplotype relative risk (supplementary text in extended data (Noyes, 2021b)).

The power analysis found the number of families that would be required to detect haplotype relative risks (HRR) of 1.5, 2 and 3 with haplotype allele frequencies from 0.05 to 0.95 after correction for the 378 haplotype blocks being tested (extended data Figure 2A (Noyes, 2021b)). The analysis showed that 250 families would provide at least 80% power to detect a HRR of 2 for haplotypes with allele frequencies between 16% and 72%. At 80% of haplotype blocks over 50% of chromosomes were on a haplotype with a frequency in the informative range (extended data Figure 2B (Noyes, 2021b)). However, at 46 out of the 378 loci no haplotypes had allele frequencies in this range (extended data Figure 2C (Noyes, 2021b)) and therefore only large HRR (HRR > 2) are likely to be detectable at these loci. In summary 250 families will provide at least 80% power to detect HRR > 2 at the 60% of blocks that have haplotypes with allele frequencies between 16% and 72%. The number of alleles and allele frequencies in a haplotype block can be controlled by changing the block length. We used a standard block length of four SNP loci for our simulation but the optimum number for maximum potential power will vary between loci depending on allele frequencies and local linkage disequilibrium. Prior to running the association tests, we will use the genotype data to optimise the sets of haplotype blocks within each gene to maximise theoretical power to detect associations.

Consent to publish. All authors will approve the final manuscript before publication. No institutional consent is required for publication.

Data management. Study documents will be kept securely in the institution which will conduct the research, i.e. University of Dschang (Cameroon samples), INRB (DRC), Makerere University(Uganda samples) and University Jean Lorougnon
Guédé University (Ivory Coast). These will be in physical lockable cabinets for forms and password protected databases with only study staff and investigators having access. All data will be anonymized before publication in appropriate scientific journals. Accompanying genotype data will also be anonymized and will be deposited in an open access depository.

Data will be collected in standardised forms as shown in supplementary data. It will be entered in Excel sheets and SQL database with range checks. The variables in the case report forms will be precoded for categorical variables to allow proper analysis. Physical data forms will be stored securely. Only the study investigators and project staff involved in generating the data will have access to the data.

**Dissemination of study outcomes.** No actionable information is expected from the association analysis and individual results will not be returned to participants. General feedback will be shared with study participants and community leaders at public meetings and results shared with policy stakeholders including officials of the National Schistosomiasis Control Programs and Ministries of Health. Sponsors of the research will receive periodic reports on study progress. Analyses will be performed separately for each study population and published independently so that national authorities can have a clear understanding of the genetic risk factors for schistosomiasis in their countries. A meta-analysis of the combined data will also be undertaken and published separately.

**Study 2:** Case-control association study

**Study site: Lake Albert region, Uganda.** The major difference between Study 1 and Study 2 is the study design. They therefore share the schistosomiasis specific methods described above for study 1; the CCA test, Kato Katz, DNA extraction and genotyping and the panel of genes to be tested. Study 2 will differ from study 1 in recruitment procedures including inclusion criteria, definition of cases and controls and data analysis methods. Below we describe what will be the different in study 2.

**Recruitment and case definition.** In the study site, the population will be screened using the CCA rapid test with results scored visually as trace, +1, +2, +3 (see section on CCA above) (Casacuberta-Partal et al., 2019). All individuals will be invited to give a blood sample for DNA extraction and plasma for subsequent worm burden quantification by CAA.

Extreme phenotype sampling will be done, where only the genotypes of the top 25% and bottom 25% of worm burden will be analyzed as described by Li et al. (Li et al., 2019). Genotyping only the participants with extreme phenotypes reduces the genotyping cost by 50% but has very little effect on power (Li et al., 2019). To allow determination of these extreme phenotypes, worm burden will be determined on plasma using the CAA test in the laboratory at UVRI. The CAA test has been described above. Since the case-control design requires controls to be unrelated to the cases, the 25% of participants in the top and bottom tails of the CAA distributions will be included for genotyping. The samples will be genotyped using the H3A chip defined above. The genotype data will be used to filter out individuals who are first cousins or closer and share a phenotype to minimize bias.

After filtering, the target proportions of the total sample for cases and controls is expected to be at least 20% of the original population sampled. In a multiplicative model; a 20% fraction from each extreme phenotype would give 80% power to detect 35% increase in risk; for MAF 0.3 with sample size 300 (60 cases and 60 controls genotyped) (Li et al., 2019). Most effects in the classical SM1 locus QTL have been shown to be co-dominant (Abel et al., 1991; Marquet et al., 1996) and therefore a multiplicative model is appropriate.

We will therefore aim to recruit at least 500 CAA positive children in Uganda. The case-control recruitment profile is shown in Figure 2.

**Additional data recorded per participant.** The following features will be recorded at the time of sample collection as potential covariates during analysis: age, sex, fever, diarrhea, infections with other parasitic worms will be detected by microscopy as part of the KK procedure, clinical observation for distended abdomen, Mid Upper Arm Circumference (MUAC) and body mass index,BMI (weight divided by the square of height in metres). The case report is provided as part of the extended data (Noyes, 2021a).

**Data analysis.** A candidate gene case control linear regression analysis will be undertaken in Plink v1.9 and R 4.0, with haplotypes as the independent variable and CAA as the dependent variable. Results will be controlled for population structure using principal components also calculated in Plink v1.9 as covariates. In this sampling design, it is critical to control for population structure (Panarella & Burkett, 2019), and genotyping on the whole genome H3A chip will provide sufficient data for a calculation of principal components for this purpose. Age, sex, MUAC and BMI will also be used as additional covariates. Procedures for the KK test will be the same as those of study 1.

**Dissemination of study outcomes.** Study participants will immediately receive their results for testing of schistosomiasis and will be advised on control measures by the health team. Since the national control programs are also involved in the study, they will have access to the results of the study for use in programmatic operations and policy decisions.

**Selection of candidate genes to be tested**

The set of candidate genes that we will test (Table 1) is based on our recent review of all previous studies of genetic variants associated with schistosomiasis infection intensity (Mewamba et al., 2021). We included candidate genes based on three sets of overlapping criteria:

1. Eleven genes that have been found associated with schistosomiasis in other populations (IFNG, IL10, IL13,
**Figure 2. Study profile for the case-control study.** POC-CCA=point of care-circulating cathodic antigen.

2) Five genes in schistosomiasis quantitative trait loci (QTL) that are known to participate in the response to schistosomiasis infection but have not previously been tested in candidate gene studies (*IL17A, IL17B, IL17F, IL6R, IL12B*)

3) Eight genes in the Th17 pathway (*IL1A, IL1B, TGFBI, IL6, IL21, IL23A, IL25, IL17RA*)

SNP in only four of the eleven previously tested genes in Table 1 have been found associated with infection with schistosomes in more than one population, these genes are indicated by multiple citations in Table 1. It is important to replicate the findings for the other genes since the earlier studies were generally small and candidate gene studies are known to be poorly reproducible (Hirschhorn et al., 2002).

Four quantitative trait loci (QTL) associated with *S. mansoni* egg count have been discovered (Marquet et al., 1996; Marquet et al., 1999; Müller-Myhsok et al., 1997; Zinn-Justin et al., 2001). These QTL were discovered before the human genome provided lists of genes in these regions. We systematically scanned these regions for genes that have been studied in the context of schistosomiasis (Mewamba et al., 2021). Genes in QTL with strong evidence for involvement in the response to infection have been included in this study. These genes are annotated with the QTL location in Table 1.

Three of the four QTL regions contained genes in the Th17 pathway. The Th17 system has two main functions: it regulates
the clearance of extracellular pathogens, it also helps the B cells to induce tissue inflammation (Patel & Kuchroo, 2015). Although the Th\textsubscript{17} system is known to be involved in the response to schistosomiasis (Larkin et al., 2012; Mbow et al., 2013), no studies of the genetics of Th\textsubscript{17} pathway genes in human schistosomiasis have been conducted so far. We therefore included Th\textsubscript{17} genes in the QTL regions in our study (Table 1) as well as important Th\textsubscript{17} genes that were outside the QTL (IL1A, IL1B, IL17RA, IL21, IL23, IL25, IL6, TGFB1).

By testing for association with schistosomiasis in four different populations we will be able to identify genes which have variants which contribute to the heaviest worm burdens across Africa. Other genes may be of local importance or previously observed associations might be false positives as a consequence of sampling from stratified populations. Genetic data has recently been used to compile risk scores for developing schistosomiasis related sever liver disease (Dessein et al., 2020). With the data from this study, it may be possible to develop a similar risk score for acquiring high worm burdens.

**Discussion**

There is abundant evidence for genetic contributions to the risk of acquiring schistosomiasis and to having particularly heavy worm burdens (Dessein et al., 2020; Mewamba et al., 2021). Some of this evidence has come from candidate gene studies that tested for association with genes that were known to be involved in the response to infection, but these studies have rarely been replicated and, where they have, the results have often been inconsistent. Other evidence has come from mapping studies that have identified QTL, regions of the genome that contain one or more genes with variants that change the risk of schistosomiasis. However, in most cases, no genes within these regions have been tested for association with disease. We have systematically surveyed these genes for plausible candidate genes which are known to participate in the response to infection (Mewamba et al., 2021) and observed that Th\textsubscript{17} pathway genes were found within the majority of these regions.

We will genotype the samples on the H3Africa Whole Genome SNP chip making it possible to scan for associations with any gene in the genome in a Genome Wide Association Study (GWAS). However large numbers of samples are required for a GWAS to compensate for the large number of tests being conducted and, since we only expect to collect 100 families per population for family-based analyses and 100 cases and 100 controls for the case control study, we would only have the power to detect exceptionally large relative risks in a GWAS. By declaring the list of candidate genes in advance of analyzing the data we can establish a much more limited set of hypotheses which we will have the power to test with the number of samples available.

**Study status**

The study is still recruiting participants. The current progress is: Cameroon (100 families consisting of 411 individuals); Ivory Coast (10 families consisting of 39 individuals); Democratic Republic of Congo (14 families consisting of 40 individuals); Uganda (LaVIISWA; previous family based collection, 40 families; Albertine case control collection: 600 individuals).

**Data availability**

**Underlying data**

No data are associated with this article.

**Extended data**

Harvard Datavers: Consent forms and CRFs for family based or case control schisto studies. [https://doi.org/10.7910/DVN/6OLLGW](https://doi.org/10.7910/DVN/6OLLGW) (Noyes, 2021a).

This project contains the following extended data:

- CAM_autorisation_recherche_schisto.pdf (Cameroon health ministry authorisation for research)
- CAM_Clairence\_ethique\_schistosomiase.pdf (Cameroon ethics review clearance)
- CAM_Consent\_form\_Anglais.pdf (Cameroon consent form in English)
- CAM_Consent\_form\_french.pdf (Cameroon Consent form in French)
- CAM\_Information\_sheet.pdf (Cameroon information sheet)
- CIV\_Assent\_form.docx (Ivory Coast Assent form English)
- CIV\_Consentements\_Projet\_TrypanoGen+.docx (Ivory Coast information sheet, consent forms in French)
- CIV\_Informed\_Consent\_Form.docx (Ivory Coast Consent form in English)
- DRC\_collective\_consentform\_and\_CRFs.docx (Democratic Republic of Congo Consent form and case report form)
- DRC\_Fiche\_de\_Consentement\_et\_Information.docx (DRC information sheet)
- DRC\_Fiche\_de\_Consentement\_Personnel.docx (DRC personal consent form)
- UGA\_LaVIISWA\_consent\_form.pdf (Uganda LaVIISWA study consent form: Refer for future use allowing genetic studies)
- UGA\_SCHISTO\_Assent.pdf (Uganda assent form for the case-control study)
- UGA\_Schisto\_Consent.pdf (Uganda case control study consent form)
- UGA\_SCHISTO\_CRF.pdf (Uganda case control study case report form)
- UGA\_Schisto\_Sample\_Storage.pdf (Uganda case control study sample storage consent)
Harvard Dataverse: Haplotype Genotyping. [https://doi.org/10.7910/DVN/OVQPCO (Noyes, 2021b)].

This project contains the following extended data:

- Files for modelling the effect of using haplotypes for transmission disequilibrium test
  - SupplementaryMaterial_Association_analysis_with_haplotypes.pdf (Description of association analysis with haplotypes and the power to detect different haplotype relative risks given different haplotype allele frequencies)
  - SupplementaryTables_1KG_African_haplotype_blocks.xlsx (Excel file containing lists of haplotype blocks identified in candidate genes in One Thousand genomes African samples, together with the number of samples required to have 80% power to detect Haplotype relative Risk of 1.5 or 2.0 or 3.0. Also, a worksheet with details of each haplotype in each block)

- Software
  - ReadMe.txt (Description of how to run the pipeline)
  - makeHaplotypes.pl (Perl script called by runBigLD.sh to obtain the SNP in each haplotype block, identify all the haplotypes in a block and count their frequencies. It outputs Summary.HapStats.1kg.h3a.POP.txt containing summary statistics on each haplotype block in each gene and counts of observations of each haplotype. POP is a population name)
  - plotBlocks.R (Rscript to make plots of haplotype blocks for each 1000 genomes African population)
  - replaceFamIds.pl (Perl script to modify 1000 genome sample ids so that they ids include a three-letter population identifier. Uses ids and population names in OneKg.pops. Takes a fam file with 1000 genome ids as input parameter)
  - runBigLD.R (Rscript that uses the BigLD R package to identify haplotype blocks and output a linkage disequilibrium heatmap and a text file of haplotype block co-ordinates. The text file is subsequently deleted)
  - runBigLD.sh (Wrapper shell script that prepares input files for runBigLD.R, calls runBigLD.R and also calls makeHaplotypes.pl to process runBigLD.R output)

- Data files used in input
  - OneKg.pops (Text file with all 1000 genome ids and the populations to which they belong. Used by replaceFamIds.pl)
  - exons.ensGRCh37.txt (Co-ordinates of exons for use by plotBlocks.R)
  - getExons.sh (shell script to extract exons for just genes of interest from a larger mart_export.txt (not provided) file which should be downloaded from Biomart. Note the script makes assumptions about the columns in which data will be found and these should be adjusted according to the mart_export.txt file used)
  - SchistoCandGenes.1kg.h3a.bed (Plink bed file with genotypes of SNP that are present in both the 1,000 genomes dataset and the Illumina H3Africa genotyping chip and that are in the candidate genes analysed)
  - SchistoCandGenes.1kg.h3a.bim (Plink bim file with identifiers of SNP that are present in both the 1,000 genomes dataset and the Illumina H3Africa genotyping chip and that are in the candidate genes analysed)
  - SchistoCandGenes.1kg.h3a.fam (Plink fam file with identifiers for 1000 genomes African samples)

- Output from simulations for each country and combined
  - All.HapsStats.1kg.h3a.ALL.txt (Sequence of each haplotype in each haplotype block in each candidate gene and counts of observations of each haplotype for all populations (GWD, MSL, YRI, ESN, LWK))
  - All.HapsStats.1kg.h3a.ESN.txt (Sequence of each haplotype in each haplotype block in each candidate gene and counts of observations of each haplotype for ESN population)
  - All.HapsStats.1kg.h3a.GWD.txt (Sequence of each haplotype in each haplotype block in each candidate gene and counts of observations of each haplotype for GWD population)
  - All.HapsStats.1kg.h3a.LWK.txt (Sequence of each haplotype in each haplotype block in each candidate gene and counts of observations of each haplotype for LWK population)
  - All.HapsStats.1kg.h3a.MSL.txt (Sequence of each haplotype in each haplotype block in each candidate gene and counts of observations of each haplotype for MSL population)
  - All.HapsStats.1kg.h3a.YRI.txt (Sequence of each haplotype in each haplotype block in each candidate gene and counts of observations of each haplotype for YRI population)
  - GeneHapBlockPlots.pdf (Output from plotBlocks.R)
  - Heatmaps.tif.tar.gz (Heatmaps showing linkage disequilibrium and haplotype blocks within each gene. The heatmaps are output from the runBigLD.R Rscript)
  - Summary.HapStats.1kg.h3a.ALL.txt (Summary statistics on each haplotype block in all populations)
Eriksson J, Reimert CM, Kabatereine NB, et al.: The 434 (G→C) polymorphism within the coding sequence of Eosinophil Cationic Protein (ECP) correlates with the natural course of Schistosoma mansoni infection. Int J Parasitol. 2007; 37(12): 1359–1366. Published Abstract | Publisher Full Text | Free Full Text
Glinz D, Silué KD, Knopp S, et al.: The 434 (G→C) polymorphism within the coding sequence of Eosinophil Cationic Protein (ECP) correlates with the natural course of Schistosoma mansoni infection. Int J Parasitol. 2007; 37(12): 1359–1366. Published Abstract | Publisher Full Text | Free Full Text
The manuscript *Candidate gene family-based and case-control studies of susceptibility to high Schistosoma mansoni worm burden in African children: a protocol [version 1; peer review: awaiting peer review]* by Nyangiri *et al.* aims to test 24 candidate genes, mainly in the Th2 and Th17 pathways, for association with *S. mansoni* infection intensity in four African countries (Ivory Coast, Cameroon, Uganda and Democratic Republic of Congo), using family based and case-control approaches. Regarding the dissimilarity observed in Schistosomiasis infection and related morbidity, this study is relevant for the field and could help for a better management of Schistosomiasis in endemic areas.

The authors have done a preliminary work by reviewing all genetic factors that control Schistosomiasis infection (Mewamba *et al.* 2021, Frontiers in Immunology) and seem to have the baseline knowledge to conduct the proposed study. The objectives of the study are clearly established and the description of the Methodology appropriate. The respect of the ethic is well described and the authorization needed gathered. The proposed statistics and meta-analysis are comprehensive.

However, the following suggestions will help improve the overall quality of the results.

Since the authors intend to include school children, they will consider obtaining authorization from Ministries of Education.

In addition to CCA and CAA, the authors should consider taking several stool samples (2 to 3) from consecutive days for KK analysis for an increased sensitivity using the resulting geometric mean of burden intensity. In fact, the CCA test could easily lead to false positive results in endemic areas, and the CAA is a good correlate for worm burden and not of eggs burden (as CAA levels indeed reflect dynamics of worm burdens and not of egg excretion or worm fecundity). KK remains the appropriate technique to accurately determine the egg burden. Of note, Schistosomiasis pathology is eggs (and not worms) driven. CCA and CAA can be useful in determining controls (true negative with all techniques) in the case-controls studies in Uganda.
The authors propose to use the top 25% positive participants as participants with high burden. Considering the WHO classification for *S. mansoni* infection (light < 100; 100 < Moderate <400 or heavy > 400), what would be the contingency plan if participants in the top 25% have a burden lower than 400 EPG? Or 100EPG?

The Figure 1 is quite confusing. The authors should consider revising it. Also, excluded samples should be mentioned. In addition, participants should be screened for clinical signs of anemia and excluded prior to blood collection.

The authors should consider screening for other confections such as malaria etc. - generally co-endemic in these regions, as co-infections could affect the susceptibility of Schistosomiasis infection. Risk factors of susceptibility/Resistance to Schistosomiasis infection will be valuable information to have.

It is not mentioned if the participants will be treated with Praziquantel at the end of the study.

For the case-control study, the authors should correct for socio-demographic data to ensure minimal variation across the groups. For example, to fully attribute the difference in infection intensity to the genetics, the authors should make sure factors like age; gender; BMI etc. are not different between cases and controls. This will aid in minimizing confounding factors.

**Is the rationale for, and objectives of, the study clearly described?**
Yes

**Is the study design appropriate for the research question?**
Yes

**Are sufficient details of the methods provided to allow replication by others?**
Partly

**Are the datasets clearly presented in a useable and accessible format?**
Not applicable

*Competing Interests:* No competing interests were disclosed.

*Reviewer Expertise:* Infectious Diseases and Fibrosis

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.