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Polymorphisms in IL-10 (-1082) and IFN-γ, (+874) cytokine genes associated with resistance or susceptibility to *Schistosoma haematobium* infection in primary school children of Mount Darwin, Zimbabwe

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The promoter region of human interleukin-10 (IL-10) gene is highly polymorphic while the first intronic region of interferon gamma (IFN-γ) gene is also highly polymorphic. These polymorphisms are associated with susceptibility or resistance to *Schistosoma haematobium* infection. Schistosomiasis is known to be a highly inflammatory disease that requires the delicate balance of pro- and anti-inflammatory cytokines. The polymorphisms are associated with low, moderate or high cytokine production resulting in exacerbation of the infection leading to pathological severity. Urine filtration technique was used for diagnosis the *S. haematobium*. Whole blood samples were collected from 400 children aged between 6 to 13 years. Molecular determination of polymorphism related to resistance or susceptibility to infection was performed using the allele-specific polymerase chain reaction. SNPs in the IL-10 and IFN-γ cytokine genes were examined in blood samples from 400 school-aged children. Schistosomiasis was detected in 49.8% (199). For IFN-γ +874A/T, the distribution of TT, TA and AA was 7, 41 and 51% respectively. An analysis of the polymorphisms on IL-10 -1082G/A showed that most of the samples were heterozygous (47% GA) whereas AA (32%) and GG (21%) were monozygous. SNPs within the promoter region of IL-10 gene and in the intronic region of IFN-γ have been associated with altered profiles of circulating IL-10 and IFN-γ. Our findings suggest that IL-10 and IFN-γ polymorphisms participate in the progression of schistosomiasis rather than in its initial development in school aged children. It is recommended to study more polymorphisms in the other cytokines implicated in schistosomiasis.

Key words: Polymorphism, cytokine, *Schistosoma haematobium*, interleukin-10, interferon gamma.

INTRODUCTION

Digenetic trematodes of the genus *Schistosoma* are responsible for the spread of schistosomiasis (Rowel et al., 2015). Urogenital schistosomiasis can include symptoms such as haematuria, anaemia in the acute phase, fibrosis of the bladder and ureter as well as kidney damage in the later stages of infection (Person et al.,

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2016). Schistosomiasis is second in importance to malaria among the major tropical parasitic diseases and is endemic in at least 76 tropical and subtropical countries (Gasim et al., 2015). The high prevalence of the disease in the sub Saharan Africa is due to poverty, lack of preventative measures, health care and safe water and sanitation facilities. Infection with *Schistosoma haematobium* leads to severe urinary schistosomiasis in the majority of the individuals. Schistosomiasis is an infection of the intestinal or urinary system by one or more several species of *Schistosoma* (Chitsulo et al., 2000). *S. haematobium* and *Schistosoma mansoni* are of medical importance in Zimbabwe with *S. haematobium* being the most prevalent (Ndhlovu, 1994; Ministry of Health and Child Welfare, 2010). Schistosomiasis also causes anaemia by inducing pro-inflammatory cytokine-mediated dyserythropoiesis, a common feature seen in anaemia associated with inflammation (Leenstra et al., 2006). Available tools to identify point-of-care diagnostics for morbidity in the pre-school age group during urogenital schistosomiasis still need to be validated (Wami et al., 2015). The pro-inflammatory cytokines such as tumour necrosis factor α (TNF- α), interleukin-1 b (IL-1b), IL-6 regulate the acute phase response which has detrimental effects if acute phase proteins such as C-reactive protein are produced continuously especially during infection (Coutinho et al., 2006). Praziquantel has been used to treat a variety of human trematode infections and is currently the drug of choice for schistosomiasis (Aragon et al., 2009).

Protection against infection has been reported to be provided by some components of the immune responses emanating from exposure to infectious agents. Some protection has been alluded to presence of antibodies, while it is believed that cytokines could be the major player in driving establishment of the protective immunity. Protective immunity to schistosomes is seen to develop slowly. Praziquantel boosts responses in IgE, IgG1 and essential protective cytokines. Inflammatory cytokines such as TNF-α, IL-1, IFN-γ, IL-6 and IL-10 are highly elevated in parasitic infections (Grau et al., 1989; Kwiatkowski et al., 2000). The pathogenesis of schistosomiasis is complex and most likely entails immunologic and non-immunologic mechanisms. The major cytokines implicated in schistosomiasis pathology include TNF-α, TNF-β, IL-10, IL-1, IL-6 and IFN-γ. TNF-α at low concentrations has a positive useful function in the immune system but an adverse effect when released in excess. The level of each cytokine by each individual is a constant feature which is genetically predetermined (Mduluza et al., 2013). Patients with elevated levels of TNF-α soluble receptor had an increased risk of dying from schistosomiasis. Polymorphisms and certain genetic diseases have been found to confer some protection against schistosomiasis.

Protection of individuals by their genetic makeup is referred to as balanced polymorphism. Some people live in endemic areas but are still found to be resistant to *S. haematobium* infection. These individuals who are resistant to schistosomiasis have been found to carry certain genes which offer them protection against the disease. The level of each cytokine produced by each individual is a constant feature which is genetically predetermined. As a result, cytokines play a central role in modulating the diseases outcome including immuno-pathology. Any effect leading to low or increased production of these delicate mediators could lead into immunological failure to infection. While these mediators are prone to mutation occurring sometimes as a single base displacement, that may result in low or over production of the mediators leading to immuno-pathological development. There are no vaccines as yet for schistosomiasis that demands more work to unravel the host-parasite relationship as correlates of protective immunity. While some individuals living in endemic areas resist infection and re-infection. Molecular characterisation of the key cytokines as markers of susceptibility or resistance to infection may provide useful pointers towards vaccine development, and even control of schistosomiasis in young children who suffer much of the infection effects. The aim of this study was therefore to determine the influence of selected cytokines and corresponding mutations on resistance and or susceptibility to *S. haematobium* infection.

MATERIALS AND METHODS

Study site and participants

The work was carried out among 400 school children of age 6 – 13 attending Bemberi Primary School in Mount Darwin district, Mashonaland Central Province in Zimbabwe. One hundred and ninety nine infected and 112 uninfected voluntary blood donors from the same population were recruited into the study. The target district was reported to have high prevalence and infection rate. All the infected participants were treated with the standard dose of 40 mg/kg of praziquantel.

Study design, inclusion and exclusion criteria

A school-based cross-sectional study that involved screening of study participants for *S. haematobium* was conducted. Before the recruitment of children into the study, the principal investigators held extensive meetings with the community elders and the school heads. The objectives of the study were explained to both the parents and the school heads and the participation required in the study were indicated. Health education regarding schistosomiasis and its impact to individuals and the community as a whole was explained. This was part of the longitudinal study investigating the distribution of mixed infection with schistosomes, soil transmitted helminthes and *Plasmodium falciparum* among primary school children in remote areas in Zimbabwe. Informed consent in the local language was conducted for the individuals and their guardians to decide if they wanted to participate in the study. Each participant was requested to provide at least 5 ml of blood. Those who could not provide blood samples were excluded in the study. All children attending the primary school were eligible. Data that include age, gender and the villages where the participants lived was recorded.

Table 1. Primer sequences and expected band sizes.

Cytokine	Nt	Mutation	Primers (5'- 3')	Product size (bp)
IL-10	-1082	A - G	A: TAA GGC TTC TTT GGG AG G: TAA GGC TTC TTT GGG AA Generic: TAA ATA TCC TCA AAG TTC C	258
IFN-γ	+874	A - T	T: TTCTTACAACACAAAATCAAATCT A: TTCTTACAACACAAAATCAAATCA Generic: AGG ATG TGT TCC AGG CTC CT	288

Parasitological screening

Stool samples from participants were examined for the presence of *S. mansoni* infection by Kato-Katz method as described previously (Katz and Pellegrino, 1972). The intensity of infection was classified based on the WHO criterion as light, moderate and heavy (WHO, 2010).

Blood collection and detection of *S. haematobium*

Approximately, 5 ml of venous blood was collected into EDTA tubes. This was used for DNA extraction, determination of cytokine gene single nucleotide polymorphism and plasma cytokine assays. Urinary schistosomiasis was diagnosed by the urine filtration technique. Children diagnosed positive for *S. haematobium* and *S. mansoni* were treated with praziquantel according to local schistosomiasis case management guidelines.

Cytokine profiles

Concentrations of IL-10 and IFN-γ were determined by indirect enzyme-linked immunosorbent assays (ELISA) using pairs of cytokine-specific monoclonal antibodies. All measurements were done in duplicate and the mean of the two values of optical density was used for all analyses.

Genomic DNA extraction

Blood was used for DNA extraction using the Qiagen FLEXI kit following the manufacturer's instructions. Frozen blood was thawed at 37°C with constant agitation. The DNA was quantified using a Nanodrop ND-1000 Spectrophotometer (Thermo Scientific). The sample was kept frozen at -20°C, only to be thawed just before performing polymerase chain reaction (PCR) amplifications. The purity and quantity check was used to evaluate the DNAs quality as well as integrity. The purity values were based on the A260/280 ratio was ranging from 1.7-2.2 and for concentration the minimum value tolerated was 22 ng/µl. Any sample out of range and/or below minimum values was re-extracted.

Molecular determination of resistance or susceptibility to infection

The thin walled 0.2 ml PCR tubes and lid chains were obtained from Inqaba. Briefly, two different 5' primers respectively specific wild type and mutant separately mixed to a 3' generic, with a final concentration of polymerase, 200 µM each deoxynucleotide, 1× reaction buffer and 0.5 µM of each specific primer mix. About 5 µl of primer mix A was added into the specific tubes for each sample, while primer mix G was added was added into the G tube. The same method was done for IFN-γ, where about 5 µl of primer mix A (mixture of primer A and generic primer) and primer mix C (mixture of generic primer and primer C). Five microliters of about 30-150 ng of template DNA was finally added into the specific tubes and the tubes loaded onto a thermocycler (PXE 0.2 thermocycler). ARMS-PCR for amplification of IL-10 (-1082 G/A) and IFN-α (+874A/T) alleles were used under the following conditions: amplification consisted of a 5 min denaturation step at 94°C; 30 cycles of 1 min at 94°C and 1 min at 60°C and 1 min at 72°C; and 5 min at 72°C, followed by cooling to 4°C (Table 1).

Detection of amplicons

The ARMS-PCR products were detected by electrophoresis on 2% agarose gel prepared in 1× TBE (45 mM Tris-borate, 1 mM EDTA) buffer at pH 8.3 and stained with ethidium bromide (0.5 µg/ml). The products of wild type primer and mutant primer were loaded next to each other. Electrophoresis was carried out at constant voltage of 90-120V for 45 min. The 100 bp DNA ladder was included. The gels were viewed on a UV trans-illuminator and mages printed. The presence or absence of mutations was scored from the images and the printouts.

Statistical methods

Pearson's chi-squared test was used to analyse the distribution of the SNPs and the correlation with cytokine levels. The probability values (P values) and 95% confidence intervals (CI) were calculated. P values less than 0.05 were considered significant.

RESULTS

Parasitology

Not all children were able to provide blood samples due to various reasons including religious beliefs among others. Schistosomiasis was more distributed in males than females. *S. haematobium* species were mainly found in this study area. The population under study had various levels of infection intensities but the majority fell in the moderate schistosomiasis category as determined by the quantitative diagnosis.

Table 2. Allelic frequencies obtained for the IL-10 and IFN-γ gene in the study population.

Cytokine polymorphism	Uninfected n(%)	Infected population n(%)	Total n(%)
IL-10 (-1082G)	44 (39)	94 (47)	138 (44)
IL-10 (-1082A)	68 (61)	105 (53)	173 (56)
IFN-γ (+874A)	102 (91)	18 (9)	120 (39)
IFN-γ (+874T)	97 (89)	22 (11)	119 (38)

Data are expressed as number and (percentage).

Table 3. Genotypic frequencies obtained for the IL-10 and IFN-γ gene in the study population.

Genotype distribution	Uninfected (%)n=112	Infected population (%)(n=199)	Total (%) n=311
IL-10 -1082GG	20(18)	44(22)	64 (21)
IL-10 -1082GA	47(42)	100(50)	147 (47)
IL-10 -1082AA	45(40)	55(28)	100 (32)
IFN-γ +874AA	53(47)	107(54)	160 (51)
IFN-γ +874AT	46(41)	81(41)	127 (41)
IFN-γ +874TT	13(12)	11(5)	24 (7)

Data are expressed as number and (percentage).

Effect of genotype on serum concentration of IL-10 and IFN-γ cytokines

In the study, the allele frequencies at -1082G/Aand +874A/T polymorphic sites did not differ significantly between infected and uninfected individuals. Both IL-10 -1082 G/A alleles occurred with almost the same frequency in schistosomiasis infected and schistosomiasis negative participants. Both IL-10 -1082 T/A alleles occurred with almost the same frequency in the total population (Tables 2 and 3, Figures 1 to 5).

DISCUSSION

S. haematobium infection is claimed a serious health problem in Zimbabwe and in most tropical and subtropical regions. This is because *S. haematobium* is the most virulent and prevalent in these countries (Ministry of Health, 2010). Evidence is accumulating that the pathology observed in schistosomiasis and other parasitic diseases is not caused directly by parasite products but by normal components of the immune response (Kwiatkowski, 2000) especially cytokines like IFN-γ, TGF-β, TNF-α/β, IL-1, IL-4, IL-10, IL-12 and IL-16 (Upperman et al., 2005). Cytokines induce severity in schistosomiasis and other parasitic infections with the production of free radicals that have an effect on immune cells. The specimens that were used in this study were collected from primary school children in Mount Darwin which is known to be endemic for schistosomiasis. The study population included school children whose ages were between 6-13 years. ARMS-PCR, a rapid and sensitive test was used in genotyping the two SNPs. Using ARMS-PCR a method adapted from Perrey et al., (1999), samples were genotyped for IL-10 (-1082) and IFN-γ (+874) polymorphisms. In the study, severity of infection as defined by the clinical presentation showed that the infection intensity level is related to schistosomiasis severity.

A few cases of *S. mansoni* infection were reported in the study. DNA was successfully extracted from most of the samples as most of the extracts were found to contain DNA. IL-10 is an anti-inflammatory cytokine whose levels are important in the regulation of inflammatory response. IL-10 is found to suppress the immuno-pathological effects of pro-inflammatory cytokines such as IFN-γ. IL-10 and IFN-γ polymorphism have been associated with protection or susceptibility to *S. haematobium* infection (Mduluza et al., 2013).

Rees et al. (2002) reported a two-fold increase in transcriptional activity compared to the G allele. High serum levels IL-10 are protective against schistosomiasis (Jallow et al., 2005). Distribution of IL-10 (-1082) genotypes showed a bias towards the heterozygous state. The frequency of the GA genotype was found to be

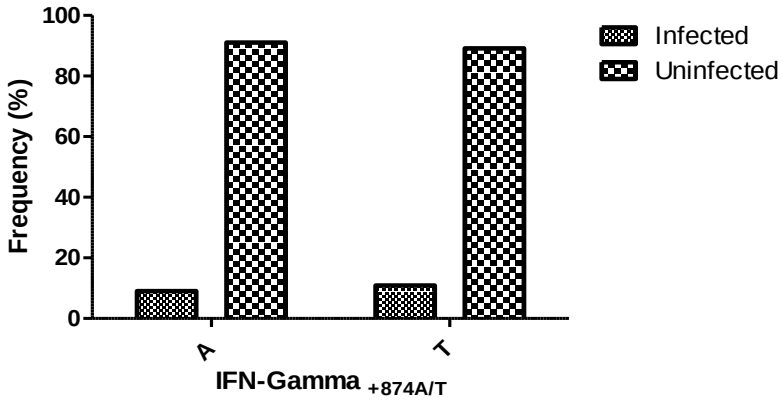

Figure 1. Total allelic frequency for IFN-γ gene +874A/T in both infected and uninfected *S. haematobium* individuals. Analysis of significance using Pearson Chi-Square test at p<0.05 showed that there is no statistical difference between the IFN-γ allelic frequencies in infected and non-infected individuals (p= 0.718).

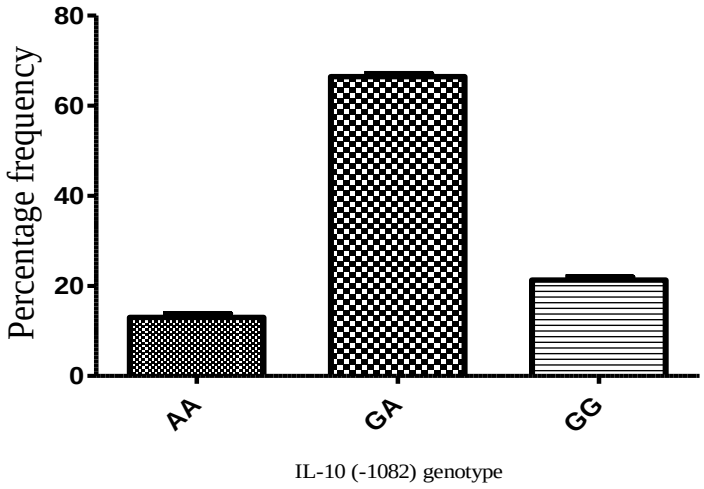

Figure 2. Distribution of IL-10 (-1082) genotypes.

47% while GG and AA occurred in frequency of 21 and 32% respectively. The distribution of IFN-γ (+874) genotypes showed a bias towards the homozygous AA state. The AA individuals are expected to be high producers of IFN-γ and are less prone to severe schistosomiasis. The genotypes of IL-10 and IFN-γ were compared from the severity of infection through infection intensity levels. The AA individuals had high infection intensity levels though there was not much difference between the moderate and high producers. The moderate GA and high GG IL-10 producers had relatively low intensity levels. This showed an association between schistosomiasis susceptibility and IL-10 polymorphism. Such host genetic factors help to explain in part why some individuals resist infection more successfully than others do. These individuals probably induce biochemical and physiological conditions that makes the growth of the parasite unfavourable. These results are consistent to what has been reported in other studies. A substitution of G by A at position -1082 of the IL-10 promoter region leads to production of low levels of IL-10 and in turn severity of schistosomiasis. The high infection intensity, the more severe the disease is. GA (moderate producers of IL-10) and GG (high producers of IL-10) genotypes at position -1082 of the IL-10 promoter region offer protection against schistosomiasis. A substitution of A by T at position +874 of the IFN-γ intronic region leads to production of high levels of IFN-γ and in low infection intensities in the study population.

The role played by IFN-γ in protection against schistosome is not yet conclusively known, but it has been said that IFN-γ activate effector cells such as

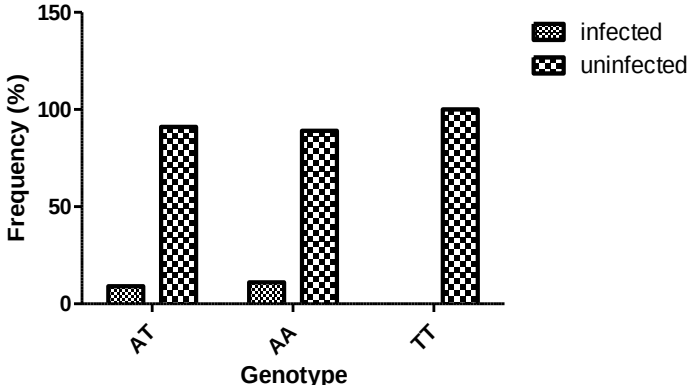

Figure 3. Genotypes for IFN-γ gene position +874A/T in *S. haematobium* infected and uninfected individuals. Analysis of statistical significance using Pearson Chi-Square test at $p<0.05$ showed that there is no statistical difference between the IFN-γ genotype in infected and non-infected individuals ($p= 0.947$).

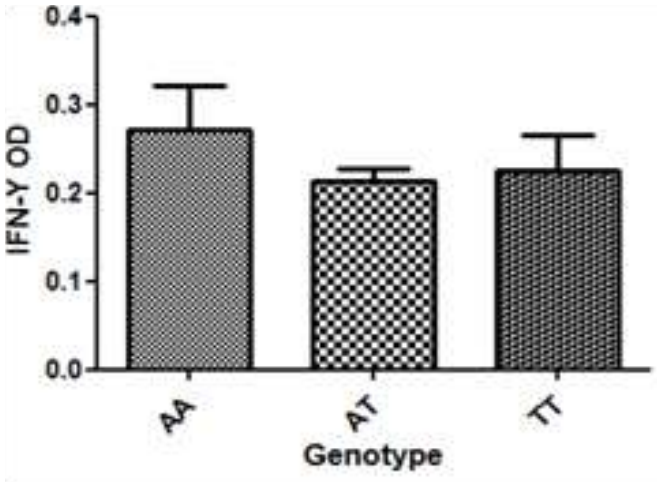

Figure 4. Comparison of IFN-γ production in genotype groups of children exposed to *S. haematobium*. The mean levels of IFN-γ production were different for each genotype. The genotype AA is seen to produce more IFN- γ than TT which also produced more IFN- γ than AT.

basophils and macrophages which produce nitric oxides and reactive oxygen species (cytotoxic agents) via the induction of the JAK/STAT pathway. The cytotoxic agents kill schistosomula (Wilson, 1998). Primary school aged children genotypes were investigated to determine if their genotypes induced an increased or decreased IFN-γ production thereby predisposing the children to susceptibility and or resistance to infection. The results of the study, Figure 5 showed that in the uninfected, children with +874 T allele produced more IFN-γ than uninfected children with the +874A allele.

A similar trend was observed in infected children, that is those with the T allele managed to produce more IFN-γ than those with the A allele. The TA genotype was the most frequently in both the uninfected and *S. haematobium* infected. We observed a statistically significant difference in the frequencies of the TA and AA genotypes. This result suggests that homozygocity for the A allele is associated with susceptibility to development of *S. haematobium*. Individuals with the AA genotype produce less IFN-γ than individuals with other genotypes. Together, these facts suggest a possible mechanism that could explain the relationship between the AA genotype and susceptibility to *S. haematobium* infection leading to severe clinical outcomes like hepatosplenomegaly, urinary tract lesions, and carcinoma in the reproductive

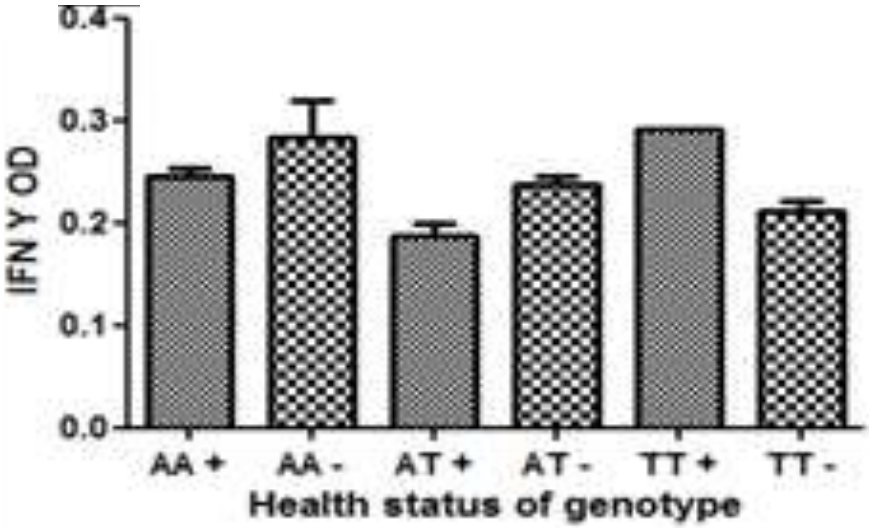

Figure 5. Comparison of IFN-γ genotype profiles in infected and uninfected children. The figure indicates that there are 3 genotypes that were observed in the study population exposed to *S. haematobium*. These genotypes are; AA, AT and TT. Analysis of significance using Pearson Chi-square test at $p< 0.05$ resulted in $P< 0.0001$ value which means that the IFN-γ levels produced by each genotype are statistically significant and different. Overall, the infected children containing the homozygous A allele produced higher levels of IFN-γ than infected children containing the T allele. The AA genotype children who were negative for Schistosomiasis produced the highest levels of IFN-γ while the AT genotype produced the least IFN-γ levels. AA^+ represents the infected with genotype AA, AA^- represents the uninfected with genotype AA. The same holds for AT^+, AT^-, TT^+, TT^-

tract. Genetic susceptibility is an important factor in the development of diseases (Chevillard et al., 2003). Collectively, our data showed that the IFN-γ +874T/A polymorphism is a determinant in the resistance or susceptibility to the development of *S. haematobium* in the study population. The advent of molecular techniques has enabled scientist to investigate diseases at a molecular level and has led to inventions of novel drugs, vaccines, and diagnostic tools for genetic and parasitic diseases. Investigation into host genetic factors in *S. haematobium* infection is of utmost importance in acquisition of knowledge of genetic based resistance and or susceptibility of individuals in areas of high *S. haematobium* endemicity. The sample size was limited by the available reagents and equipment.

Conclusion

Results from this study give insight into understanding infection patterns, protective immunity and vaccine development. Our findings suggest that IL-10 and IFN-γ polymorphisms participate in the progression of schistosomiasis rather than in its initial development in school-aged children. Cytokine polymorphisms do influence resistance and or susceptibility to schistosomiasis.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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