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Full Length Research Paper

Simple sequence repeat (SSR) marker-based DNA fingerprinting of some varieties of rice (*Oryza sativa* L.) released in Nigeria

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Over time, precise, specific and rapid identification of variety have been achieved via fingerprinting with molecular markers. In this research, 13 out of 45 screened simple sequence repeat (SSR) markers were employed to fingerprint 27 rice varieties (22 are commercially released while five are suspected duplicates from the hands of marketers with different names) in Nigeria. The SSR primer pairs (13) were polymorphic and were found to generate 81 allele distinct reproducible bands with an average of 6.233 bands per primer pair. Primer RM400 had the highest allelic frequency of 0.94 resulting from 20 alleles. The polymorphic information content (PIC) values of each primer pair ranged between 0.31 and 0.93 with an average of 0.54. The unweighted pair group method with arithmetic (UPGMA) cluster analysis helped to separate the 27 varieties into 13 major groups indicating wide range of diversity. A large number of the closely related varieties were identified by means of the fingerprinting on the basis of the polymorphic SSR primer pairs. The findings showed a broad genetic variation among the test varieties giving a first-hand insight on how related some of the commercially released varieties are and also disproving the duplicity suspected from the materials with marketers.

Key words: Cultivar, DNA, fingerprinting, rice, simple sequence repeat (SSR) marker, variety verification.

INTRODUCTION

Rice (*Oryza sativa* L.) is a staple food for more than half of the world's seven billion people (Mohanty, 2013). It is an essential food crop globally. It grows in both temperate and tropical regions of the world. Rice belongs to the family Poaceae, tribe *Oryzae* and genus *Oryza*. There are 22 species of rice; of these 22 species, 20 are wild while only two (*O. sativa* and *Oryza glaberrima*) are cultivated. *O. sativa* is the most widely grown worldwide while *O. glaberrima* is grown only in West African countries. Most rice species are diploid (2n=24), although a few are tetraploid (2n=48). Elite cultivars along with rice seeds of high-quality play a vital role in rice production, hence, it is normal for new cultivars to be developed from hybridizations between the members that constitute an elite group of genetically similar parents, and the genetic variability amount among recently developed cultivars will possibly become even smaller (Rahman et al., 2008). This makes it more challenging to clearly distinguish

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cultivars from the others having morphological features and isozyme electrophoresis patterns due to influences by environmental elements.

In rice, there are abundant microsatellites and are distributed evenly across the genome (Akagi et al., 1996; McCouch et al., 1997). Due to their codominance, ability to detect high levels of allelic diversity, and efficient assay via the polymerase chain reaction (PCR) they are valuable as genetic markers (McCouch et al., 1997). Microsatellites exist as PCR-based markers that are cost-effective to use and technically efficient and are available for rice (Chen et al., 1997; Temnykh et al., 2000). Rapid, objective and precise cultivar identification is enabled by means of fingerprinting with molecular markers, and has been certified as an efficient tool for crop germplasm characterization, management and collection. There has been a large deployment of simple sequence repeat (SSR) markers for cultivar identification and genetic analysis due to their co-dominance inheritance, abundance, reproducibility, high polymorphism, and easy to read-off by PCR (Kuleung et al., 2004; Karihaloo et al., 2015; Xie et al., 2011). Past researches on rice have enhanced the development of hundreds of microsatellite markers and a genetic map comprising so many markers for documentation and characterization. In crop species, this approach has recently been used (Ngailo et al., 2016; Rodriguez-Bonilla et al., 2014; Yada et al., 2015; Yang et al., 2015). In Bangladesh, nine soybean cultivars were identified through microsatellite markers, which have provided distinctiveness and will perhaps be protective (Islam et al., 2007). Thirteen maize cultivars were also characterized using microsatellite fingerprinting in conjunction with DUS test (Molla et al., 2007).

Rice is the second major grain crop of Nigeria after maize. Nowadays, one of the hindrances to development of rice production is the duplicity of seeds within germplasm, markets and among farmers within the country. It is therefore, very important, to fingerprint the commercial rice varieties based on molecular markers as a crucial first measure to the ambiguity presently experienced by scientists and farmers and quick identification of similar or closely-related varieties. However, no research on the fingerprinting of the main commercial rice varieties under cultivation at present has been done in Nigeria till date. Therefore, this investigation was to give a firsthand insight on how related some of the commercially released varieties of rice are and some accessions collected within the Niger State region, as a first step to backstopping duplicity of released rice varieties in Nigeria using SSR molecular markers.

MATERIALS AND METHODS

Plant

Twenty seven rice samples, five of which are collected from farmers and markets and 22 of which are from the germplasm of National Cereals Research Institute (Table 1) were used for microsatellite analysis. Seeds were collected and were germinated at aseptic condition and grown in glass house for a period of three weeks.

Genomic DNA isolation

Using the protocol described by Aljanabi and Martinez (1997) with modifications, genomic DNA was isolated from the rice seedlings. Fresh leaf samples (22-days-old) of seedling were employed as the genomic DNA source. Leaf tissues were cut into small pieces, homogenized and digested with extraction buffer (50 mM Tris-HCl, 25 mM ethylenediaminetetraacetic acid (EDTA), 300 mM NaCl and 1% sodium dodecyl sulphate (SDS), pH 8.0). After incubation for 20 min at 65°C with intermittent swirling, the mixture underwent emulsification with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1 v/v/v). DNA was subjected to precipitation using two volumes of absolute alcohol in the presence of 0.3 M sodium acetate and pelleted by centrifugation. The pellets were thereafter washed with 70% ethanol, air-dried and resuspended in a suitable volume of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH=8.0). DNA quality was further checked by means of electrophoresis in a minigel and quantification was attained using a spectrophotometer (Spectronic® Genesis™, Spectronic Instruments Inc., USA).

Microsatellite markers and PCR amplification

Thirteen out of 42 screened microsatellite primer pairs were used in the analysis (Table 2). PCR was done in a volume of 10 µl containing 50 ng template DNA, 1 µl 10X PCR buffer containing 15 mM $MgCl_2$, 0.25 mM each of the dNTPs, 0.25 µM of each primer, 1 U ampli Taq DNA polymerase (INQABA Ltd. South Africa) and an appropriate quantity of sterilized deionized water. Amplification was conduct using a thermo cycler (Thermal cycler gradient, Nyxtechnik, USA model A6) with the following program: initial denaturation at 94°C for 4 min followed by 35 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 1 min and a final cycle at 72°C for 7 min. PCR products were examined in 2% agarose gel.

Determination of microsatellite allele lengths

PCR products were separated on 6% denatured polyacrylamide gel containing 19:1 Acrylamide:Bis acrylamide and 7 M urea. Electrophoresis was carried out on Sequi Gen GT eletrophoresis cell (Bio-neer Agaro power A7020, Korea). Gels were stained with silver nitrate using the Promega Silver Sequence TM protocol (Gustavo and Grsshoff, 1994).

Data analysis

The most intensely amplified band size (in nucleotides) for each microsatellite marker was calculated on the basis of its migration in relation to the molecular weight (mw) size markers, 100 bp DNA ladder (GENEI Pvt. Ltd. Bangalore, India) using the software DNA frag Ver. 3.03 (Nash, 1991). The data were inputed into a binary matrix as discrete variables; 1 for presence and 0 for absence of the character and this data matrix was subjected to additional analysis. The Excel file containing the binary data was imported into NT Edit of NTSYS-pc 2.02J. Using SIMQUAL subroutine in SIMILARITY routine, the 0/1 matrix was used to estimate similarity as DICE coefficient. Also, to construct dendrograms based on Unweighted Pair Group Method with Arithmetic Means (UPGMA), the resultant similarity matrix was employed to infer genetic relationships and phylogeny while NTSYS-pc version 2.02 (Rohlf, 2000) and Free Tree 0.9.1.50 (Pavlicek et al., 1999) software were

Table 1. List of the 27 varieties/accessions used for the fingerprinting.

S/N	Entry	S/N	Entry
1	FARO 34	15	FARO 33
2	FARO 15	16	FARO 37
3	FARO 16	17	FARO 44
4	FARO 17	18	FARO 50
5	FARO 19	19	FARO 51
6	FARO 20	20	FARO 60
7	FARO 21	21	FARO 61
8	FARO 22	22	FARO 62
9	FARO 26	23	ISHAQ
10	FARO 27	24	NDAYIKAKOO
11	FARO 30	25	PETERGII
12	FARO 31	26	WALUYEE
13	FARO 32	27	BERUWA
14	FARO 52	-	-

Table 2. Details of the polymorphic microsatellite markers used for rice genotype identification. SSR Primers from McCouch et al. (2002).

S/N	Primer	Forward	Reverse
1	RM225	TGCCCATATGGTCTGGATG	GAAAGTGGATCAGGAAGGC
2	RM228	CTGGCCATTAGTCCTTGG	GCTTGCGGCTCTGCTTAC
3	RM251	GAATGGCAATGGCGCTAG	ATGCGGTTCAAGATTCGATC
4	RM340	GGTAAATGGACAATCCTATGGC	GACAAATATAAGGGCAGTGTGC
5	RM341	CAAGAAACCTCAATCCGAGC	CTCCTCCCGATCCCAATC
6	RM523	AAGGCATTGCAGCTAGAAGC	GCACTTGGGAGGTTTGCTAG
7	RM224	ATCGATCGATCTTCACGAGG	TGCTATAAAAGGCATTCGGG
8	RM229	CACTCACACGAACGACTGAC	CGCAGGTTCTTGTGAAATGT
9	RM256	GACAGGGAGTGATTGAAGGC	GTTGATTTCGCCAAGGGC
10	RM304	TCAAACCGGCACATATAAGAC	GATAGGGAGCTGAAGGAGATG
11	RM463	TTCCCCTCCTTTTATGGTGC	TGTTCTCCTCAGTCACTGCG
12	RM400	ACACCAGGCTACCCAAACTC	CGGAGAGATCTGACATGTGG
13	RM244	CCGACTGTTCGTCCTTATCA	CTGCTCTCGGGTGAACGT

used to calculate Jaccard's similarity coefficient.

RESULTS AND DISCUSSION

All 27 cultivars of rice were completely amplified with 13 microsatellite primer pairs. On the basis of past results (Sefc et al., 2000), primer pairs will be known as loci whereas DNA bands as alleles. All 13 microsatellite primer from the 41 screened were found to be polymorphic, showing a total of 81 alleles with an average number of 6.32 alleles per locus among the 27 rice cultivars investigated (Table 3). From the banding patterns produced by the primer pairs, primers RM 288, RM 341 and RM 400 showed that they had the highest frequency of detected alleles of 7, 7 and 20, respectively. For the presence and absence of the SSR bands, all the genotypes were scored. Polymorphic Information Content (PIC) showed the range of the different primer pairs. Out of the 27 observations made for each of the primer pairs, RM400 had the least major allelic frequency of 0.111 which explains its high allelic number and resultant high gene diversity score (0.9410) and PIC number (0.9379). Allele frequency depends on the trait investigated which reflects directly on the allelic number and diversity of a sample as suggested by Fuentes et al. (1999) in their study. However, 13 different clusters were formed from the results of the dendogram.

Genetic diversity assessment is a key component in any breeding programme that is interested in conserving and characterizing its germplasm. Results obtained from this study indicated that more genetic diversity exist

Table 3. Marker Analysis showing gene diversity among 27 Rice varieties.

S/N	Marker	Major allele frequency	Number of obs.	Allele number	Gene diversity	PIC
1	RM225	0.7407	27.0000	4.0000	0.4252	0.3963
2	RM228	0.5926	27.0000	7.0000	0.5926	0.5524
3	RM251	0.3333	27.0000	6.0000	0.7545	0.7155
4	RM340	0.7778	27.0000	5.0000	0.3813	0.3646
5	RM341	0.5926	27.0000	7.0000	0.6173	0.5944
6	RM523	0.4444	27.0000	5.0000	0.6722	0.6162
7	RM224	0.5926	27.0000	5.0000	0.5871	0.5424
8	RM229	0.5185	27.0000	5.0000	0.6584	0.6167
9	RM256	0.5185	27.0000	6.0000	0.6612	0.6213
10	RM304	0.8148	27.0000	5.0000	0.3265	0.3137
11	RM463	0.6667	27.0000	3.0000	0.4829	0.4175
12	RM400	0.1111	27.0000	20.0000	0.9410	0.9379
13	RM244	0.6296	27.0000	3.0000	0.5240	0.4593
	Mean	0.5641	27.0000	6.2308	0.5865	0.5499

among the test entries in this gene pool (Fuentes et al., 1999; Qian et al., 1995). The result shows many cluster groups of closely related genotypes (Figures 1 and 2). However, the frequency of allele shared by the accessions clearly showed the genetic diversity present among the varieties and the higher the frequency of allele shared, the wider the diversity. It is also possible that the rice accessions closely linked to the released variety are similar or same as varieties or they are products of same cross and share parentage which speaks to denote their relative closeness.

Within breeding programs, genetic diversity is affected by classical breeding. Selection raises the alleles frequency or allelic combinations with positive effects at the expense of others which eventually eliminates many essential combinations of alleles (Cao et al., 1998). In this present investigation, SSR markers were used to characterize and assess genetic diversity among 27 rice entries of which 22 are commercially released rice varieties while 5 entries were suspected lines collected from within Niger State.

A total of 13 RM primers were utilized to provide genetic diversity or similarity among 27 commercially released rice variety and all 13 RM primers demonstrated polymorphism between 27 rice cultivars. A total of 351 bands made from 27 observations per SSR marker were scored and no bands were found to be monomorphic. The paper revealed that the primer RM400 had the highest number of alleles with a very low allelic frequency and showing very high gene diversity score. RM251 followed same pattern and similar to a work reported by Akagi et al. (1996). Significantly greater allelic diversity of microsatellite markers has also been reported in a number of studies than other molecular markers (McCouch et al., 2001).

Cluster analysis was employed in grouping the varieties and in constructing a dendogram. The similarity matrix representing the DICE co-efficient was used to cluster the data with the aid of the UPGMA algorithm. The UPGMA based dendogram obtained from the binary data deduced from the DNA profiles of the samples analyzed shows how diverse the test entries were clustering them into different groups. A total of 13 distinct groups resulted out of analysis of pooled SSR marker data (Figures 1 and 2). This dendogram revealed that the genotypes that are derivatives of genetically similar type clustered more together. The Beruwa and FARO 17 clustered in a group showing genetic closeness. Entry ISHAQ, FARO 31 and FARO 62 also clustered together which is indicative of genetic closeness. However, this investigation may not conclude that these clusters means duplicity but maybe said to be genetically close and would need further investigation to authenticate certainty.

In this study, the wide range of similarity values for cultivars revealed by micro satellite markers provides greater confidence for the assessments of genetic diversity and relationships, which can be used in future breeding programs. Principle component analysis was also done to visualize genetic relationships among the elite breeding lines (Figure 2). The results were similar to UPGMA results.

Conclusion

Based on this research, the wide range of similarity values for related cultivars using microsatellites provides greater confidence for the assessment of genetic diversity and relationships. The practical approach developed in this study is useful in DNA fingerprinting.

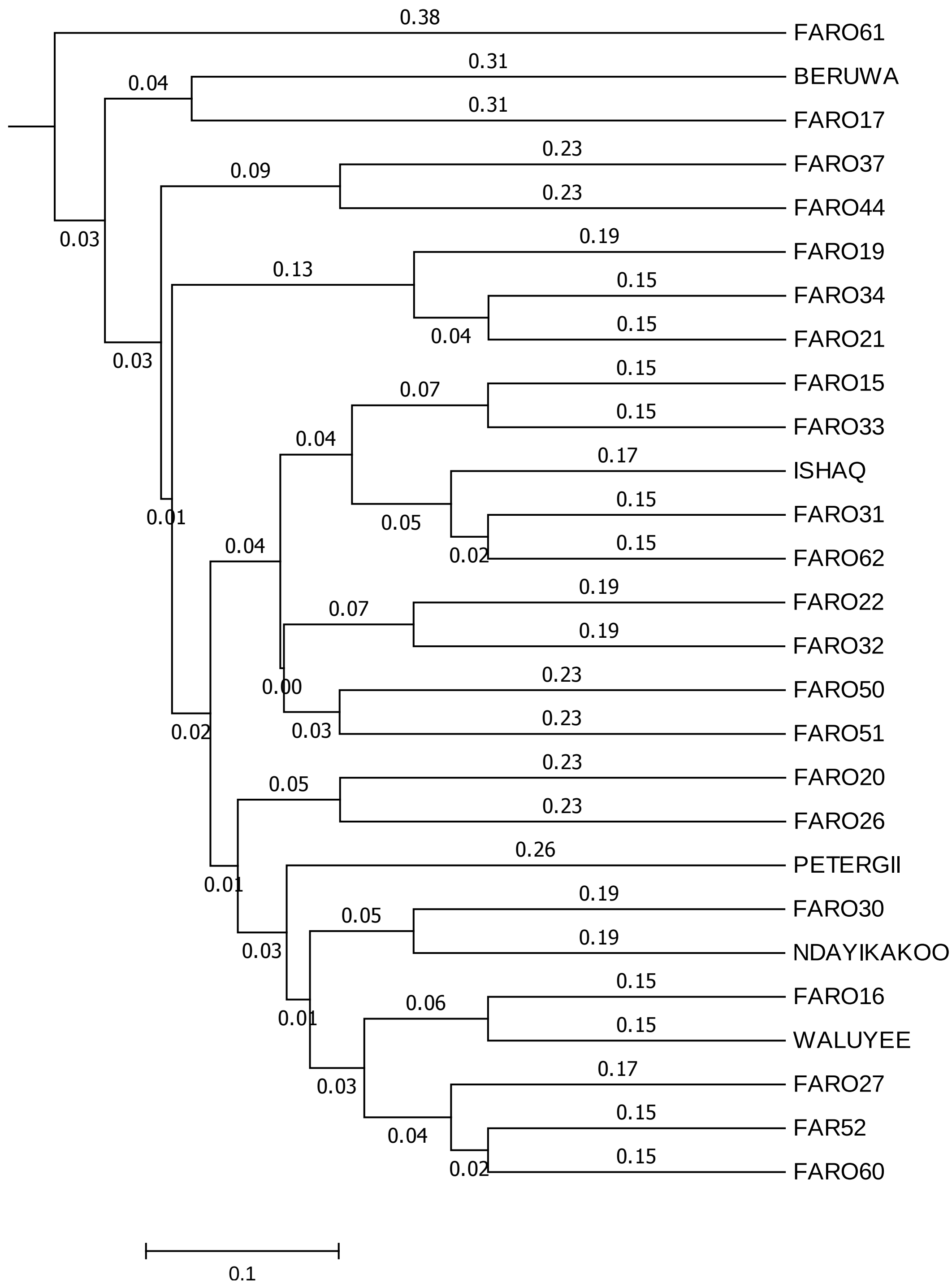

Figure 1. Cluster analysis of 27 accessions of rice showing their similarity.

Among the 45 SSR RM primers studied, 13 primers spread were found to be useful in fingerprinting of 27 commercially released rice varieties. This fingerprinting makes identification and characterization of genotype very easy and will be of greater help in background selections during back cross breeding programs.

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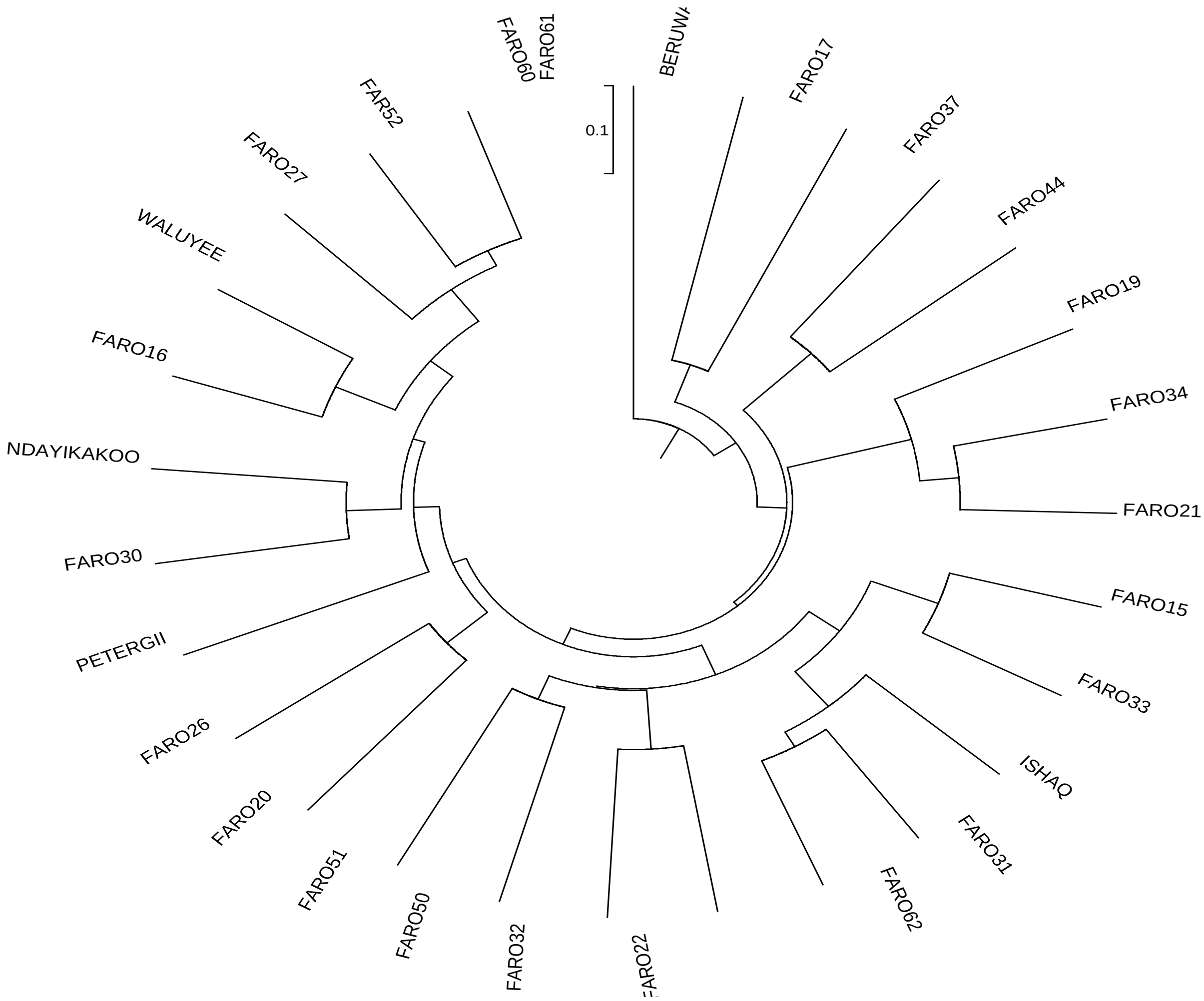

Figure 2. Dendogram showing 27 rice varieties and their relative diversity.

hours of this work and Africa rice in Ibadan, Nigeria for their assistance in getting the primers for this work.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

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

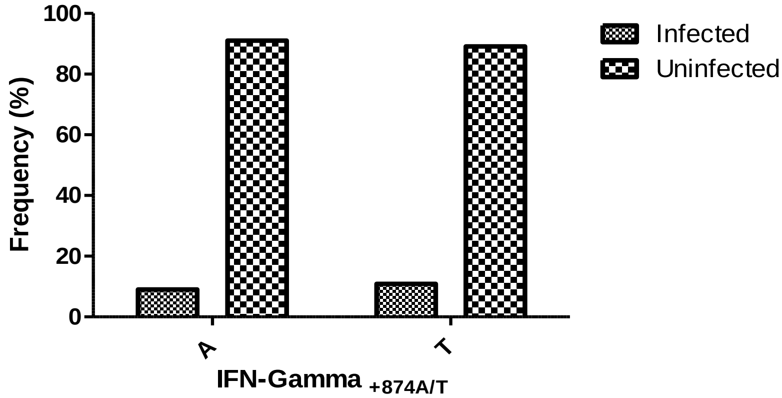

Figure 1. Total allelic frequency for IFN-γ gene +874A/T in both infected and uninfected *S. haematobium* individuals. Analysis of significance using Pearson Chi-Square test at $p<0.05$ showed that there is no statistical difference between the IFN-γ allelic frequencies in infected and non-infected individuals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

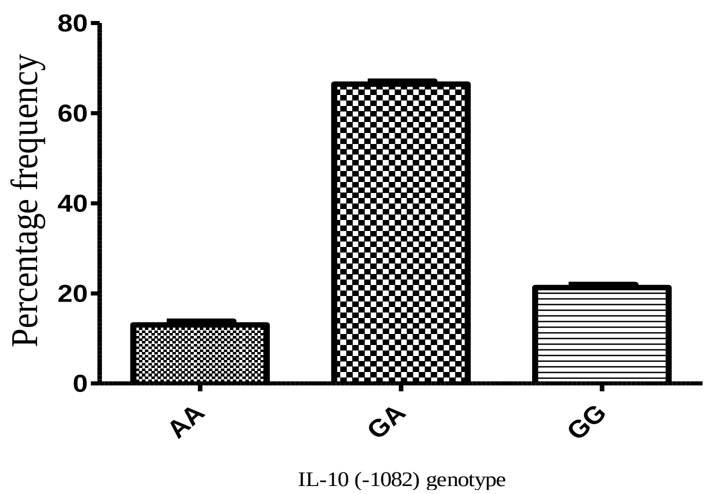

Figure 2. Distribution of IL-10 (-1082) genotypes.

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

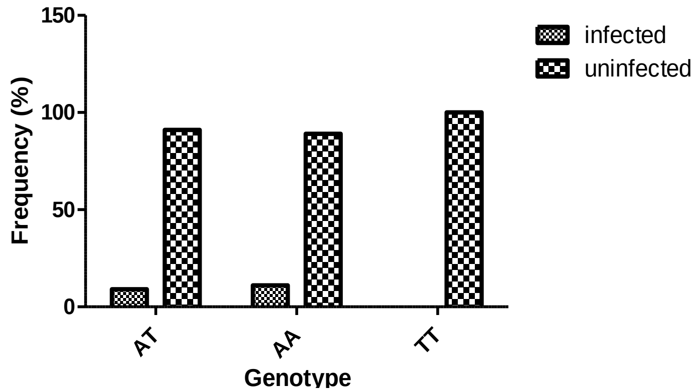

Figure 3. Genotypes for IFN-γ gene position +874A/T in *S. haematobium* infected and uninfected individuals. Analysis of statistical significance using Pearson Chi-Square test at $p<0.05$ showed that there is no statistical difference between the IFN-γ genotype in infected and non-infected individuals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

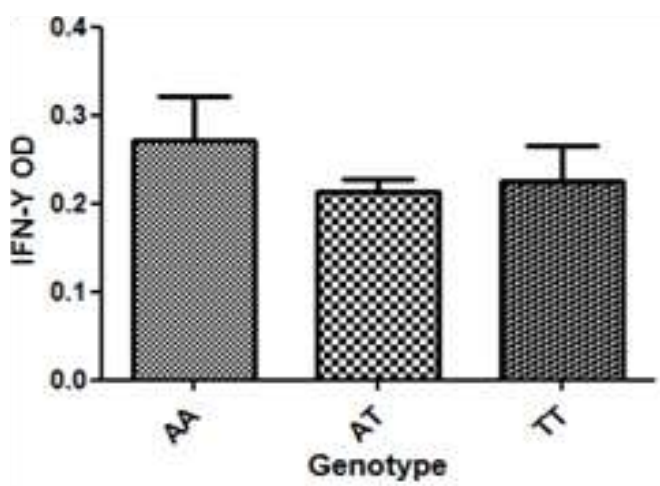

Figure 4. Comparison of IFN-γ production in genotype groups of children exposed to *S. haematobium*. The mean levels of IFN-γ production were different for each genotype. The genotype AA is seen to produce more IFN- γ than TT which also produced more IFN- γ than AT.

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

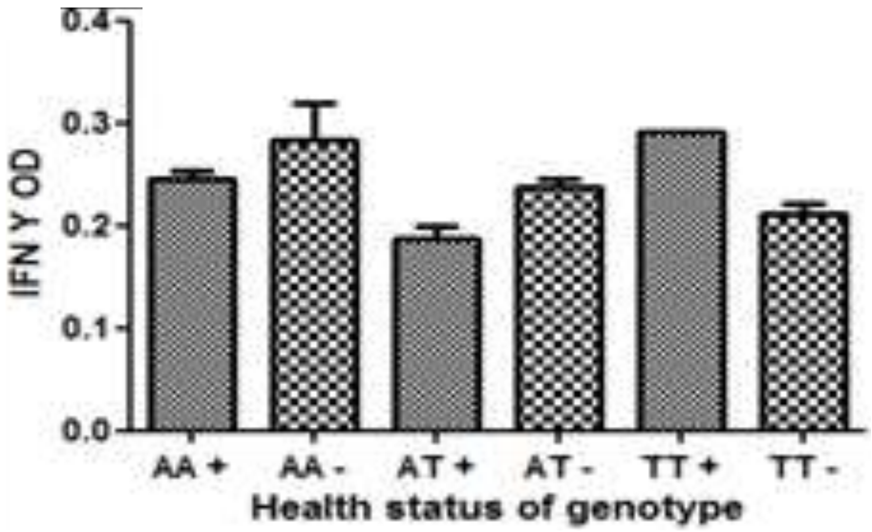

Figure 5. Comparison of IFN-γ genotype profiles in infected and uninfected children. The figure indicates that there are 3 genotypes that were observed in the study population exposed to *S. haematobium*. These genotypes are; AA, AT and TT. Analysis of significance using Pearson Chi-square test at p< 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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