Molecular Identification of Bulinus Species in Ogun State, South-West Nigeria and Observations on Snail Infections with Schistosome

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Abstract— *The study considers the distribution of a small sample of 100 Bulinus snails, across 8 localities within Ogun State, Nigerian. Snails were identified using a molecular method of fragment and restriction profiles obtained from ribosomal internal transcribed spacer (its) amplicons. The results showed that the majority of Bulinus samples tested belonged to the species Bulinustruncatus while only one was Bulinusglobosus. The use of Rsa1 restriction endonuclease to cleave the ribosomal its of Bulinus, as a method of species identification, was adopted for the majority of samples, this being a quicker and cheaper method better suited to small laboratory environments. Polymerase chain reaction (PCR) amplification of the schistosome Dra1 repeat within each of the collected Bulinus samples was employed to determine the extent and distribution of infected snails within the sample areas. Successful amplification of the Dra1 repeat demonstrated that 23% of snails were infected with schistosome*

Keywords— Bulinustruncatus, Bulinusglobosus Schistosomahaematobium, PCR-RFLP, schistosome infection.

I. INTRODUCTION

According to the World Health Organization (WHO), schistosomiasis is a worldwide public health problem affecting 200 million people in the third world. *Schistosomahaematobium*, which causes urinary schistosomiasis in humans, afflicts about 150 million people in 53 countries in Africa and the Middle East (WHO, 2002). Urinary schistosomiasis caused by *Schistosomahaematobium*, a trematode parasite, has been reported to be endemic throughout all the States of Nigeria (Mafe*et al.*, 2000; Ekpo*et al.*, 2004). Brown, (1994) also reported that freshwater pulmonate snails of the genus *Bulinus*are the intermediate hosts for *S. haematobium* and occur commonly throughout much of Africa and adjacent regions. The snails are also reported by Akogun and Akogun (Akogun*et al.*, 1996) to be more prevalent in the rural riverine areas and in areas where developments of projects to promote irrigation and provide hydroelectric power are taking place. According to Cowpear, their distributions are focal, aggregated and are usually related to the presence of several man-made impoundments, small seasonal streams, irrigation canals and ponds (Cowpear, 1973). Available diagnostic methods of schistosomiasis are search for eggs in stools or urine and detecting eggs or adult worm antigens in urine and sera of infected individuals. Sturrock*et al. (*2001) observed that surveillance techniques include monitoring schistosome eggs output by humans, human water contact activities, snail infection rates and numbers of cercariae in the water. They also observed that detection of eggs or adult worm antigens in urine and sera of infected individuals could differentiate between past and current infections with specificity close to 100%. However, they stated that the sensitivity could be low in light infections with disadvantages such as high cost, difficult approach and dependence on monoclonal antibodies. Recently a polymerase chain reaction (PCR) was developed by Rabello*etal. (*2002) for the detection of schistosome DNA in faeces. Other detection methods of schistosome infections in snails include snail crushing in search of larvae, repeated shedding of cercariae in the laboratory, detection of schistosomal antigens in snail hemolymph and lately, polymerase chain reaction (PCR) assays (Hamburger *et al.*, 1989; Hamburger *et al.*, 2001; Hamburger *et al.*, 2004; Hertel*et al.*, 2004; Driscoll *et al.*, 2005; Ten Hove*et al.*, 2008). Unlike other methods that were previously used for identifying snails with pre-patent infection, it was observed by Hamburger *et al.* (2004) that PCR can enable detection of snail infection from its very earliest stages and can identify the entire population of infected snails, regardless of whether they eventually shed cercariae.

The aim of this study, therefore, was to identify Bulinus snails involved in the transmission of *S. haematobium* in Ogun State, through polymerase chain reaction-restriction fragment length polymorphism (PCRRFLP) analysis of the ribosomal its so as to provide information that would serve as a basis for informed predictions and decisions on schistosomiasis control strategies in the State.

II. MATERIALS AND METHODS

2.1 Snail isolates

A total of 100 *Bulinus* snails were collected between May 2009 and June 2011, from 12 sites (Table 1) endemic for schistosomiasis in Ogun State, Nigeria. All the human–water contact sites in the study areas were identified and searched for snails belonging to the genus *Bulinus*. The snails were collected using a standard snail scoop, the contents washed and the Snails picked manually. Recovered snails were transported to the laboratory in pre-labelled plastic containers, rinsed, sorted and counted. Each snail was identified based on its morphological characteristics using the field guide to African freshwater snails by Kristensen, (1987).

Table.1: Sample origins and number of snails collected from each site.

Serial no	Sample origin	Number of Snails
1	Abeokuta	10
2	Ijebu Igbo	0.0
3	Abuletuntun	10
4	IjebuOgbere	8
5	Ipokia	8
6	Ado Odo/Ota	8
7	Obafemi/owode	9
8	Iloti	8
9	Shagamu	15
10	Itori	8
11	Olomore	6
12	Ikenne	10

2.2 Genomic DNA extraction from snails

Genomic DNA was extracted from each snail using a modified method of Stothard*et al.* (1996). Each snail was removed from 70% ethanol and soaked in TE buffer, pH 7.4 (10mM TrisHCl and 1mM EDTA) overnight so as to remove the remaining ethanol. Tissue from each of the snails was placed in a sterile 1.5-ml microcentrifuge tube, with 500ml of CTAB solution (0.2% 2-mercaptoethanol; 2% hexadecyltrimethyl-ammonium bromide (CTAB); 100mM Tris (hydroxymethyl) amino-methane; 16mM EDTA; 1.4M sodium chloride) and the tissue homogenized. Proteinase K solution (10ml at 20 mg/ml) was added and the digests were incubated at 55ºC for 1 h, with occasional gentle mixing. Genomic DNA was extracted from the digests by adding an equal volume of chloroform/isoamyl alcohol (24:1) to each tube. The organic and aqueous layers were gently mixed for 5 min and spun at 13,000 g for 20 min. For each sample, the upper aqueous layer was removed into another sterile micro-centrifuge tube and an equal volume of 100% ethanol was added, mixed and the whole incubated at -20ºC overnight in order to enhance DNA precipitation. The precipitate was spun at 13,000 g for 20 min and the pellet washed with 70% ethanol and spun for another 20 min. The supernatant was removed and the pellet was dried at room temperature. When completely dry, the pellet was re-suspended in 25ul of purified water and stored at 4ºC until used.

2.3 Molecular Screening of Snails for Schistosomiasis

Genomic DNA extracted from snails was subjected to PCR amplification of the schistosome*Dra1* repeat using forward primers 5'GATCTCACCTATCAGACGAAAC3' and reverse primers 5'TCACAACGATACGACCAAC 3' [9]. All the PCR amplifications were performed with the Thermal Cycler (Bio-Rad cycler) and the amplified products were visualized on 1.5% agarose gel. Photo documentation was performed with Gel Documentation.

2.4 Molecular identification of snails

Snails identification was performed by PCR amplification of the snails' ribosomal internal transcribed spacer (ITS) region using forward primer *Etts1* 5'TGCTTAAGTTCAGCGGGT3' and reverse primer *Etts2* 5'TAACAAGGTTTCCGTAGGTGAA3' (Ten Hove *et al*., 2008.) The amplification was confirmed by visualization on 1.5% agarose gel and the amplified products were digested with *RsaI*, a 6-base cutting restriction enzyme, following the method of Stothard*et al* (Ten Hove *et al*., 2008). The digest was visualized on 1.5% agarose gel followed by photo documentation using Gel Documentation.

2.4 Statistical analysis

The data was analyzed using SPSS 16.0 version. Proportional data were compared using chi-square test while other data comparison was carried out using descriptive analysis.

III. RESULTS

All snail collected from Ipokia, Ado Odo/Ota, Obafemi/Owode, Olomore and Ikenne were uninfected

while some of the snail collected from Abeokuta 1(10%), Abuletuntun 4(40%), IjebuOgbere 3(37.5%), Iloti 1(12.5%), Shagamu 5(33.3%) and Itori 5(62.5%)were infected.

Table.2: Infected and Uninfected Snails in the Studied Area

Communities	Snails collected	% infected	% uninfected
Abeokuta	10	1 (10)	9 (90)
Ijebu Igbo	0.0	0.0(0.0)	0.0(0.0)
Abuletuntun	10	4(40)	6
IjebuOgbere	8	3(37.5)	5(62.5)
Ipokia	8	0.0	8(100)
Ado Odo/Ota	8	0.0	8(100)
Obafemi/owode	9	0.0	9(100)
Iloti	8	1(12.5)	7(87.5)
Shagamu	15	5(33.3)	10(66.7)
Itori	8	5(62.5)	3(37.5)
Olomore	6	0(0.0)	6(100)
Ikenne	10	0(0.0)	10(100)

3.1 Identification of Snail

The amplification of the ITS region of the 16 snails produced bands of different sizes and the resulting fragments following digestion by restriction enzyme *Rsa1* are shown in Figure 1

Lanes – 1: size marker; 2, 4, 6, 7, 8, 9, 11, 13 and 16 while others such as 3, 10, 12, 14 and15 shows no bands 5, negative control.

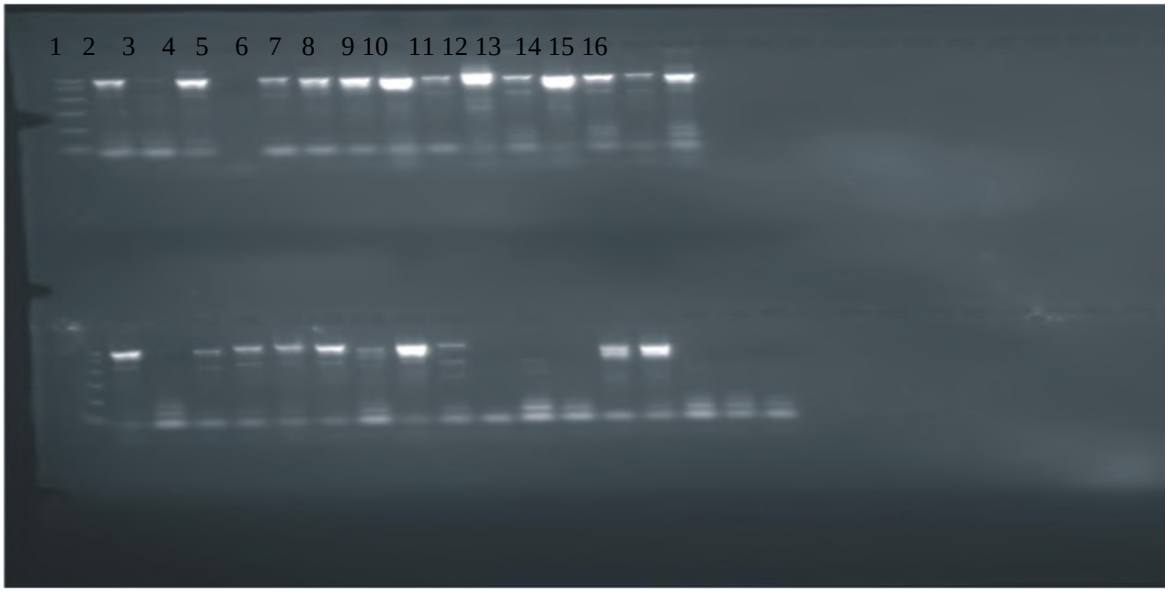

Fig.1: Agarose gel stained with ethidium bromide showing the different bands obtained from amplification of the ITS region of the 16 snails.

In figure 2,Lanes 1 positive control lane: 2–5 and 10, uninfected snails; 6 -9 and11-13 infected snails; 14-21showing weak/ambiguous profiles. Lane 25 is negative control.

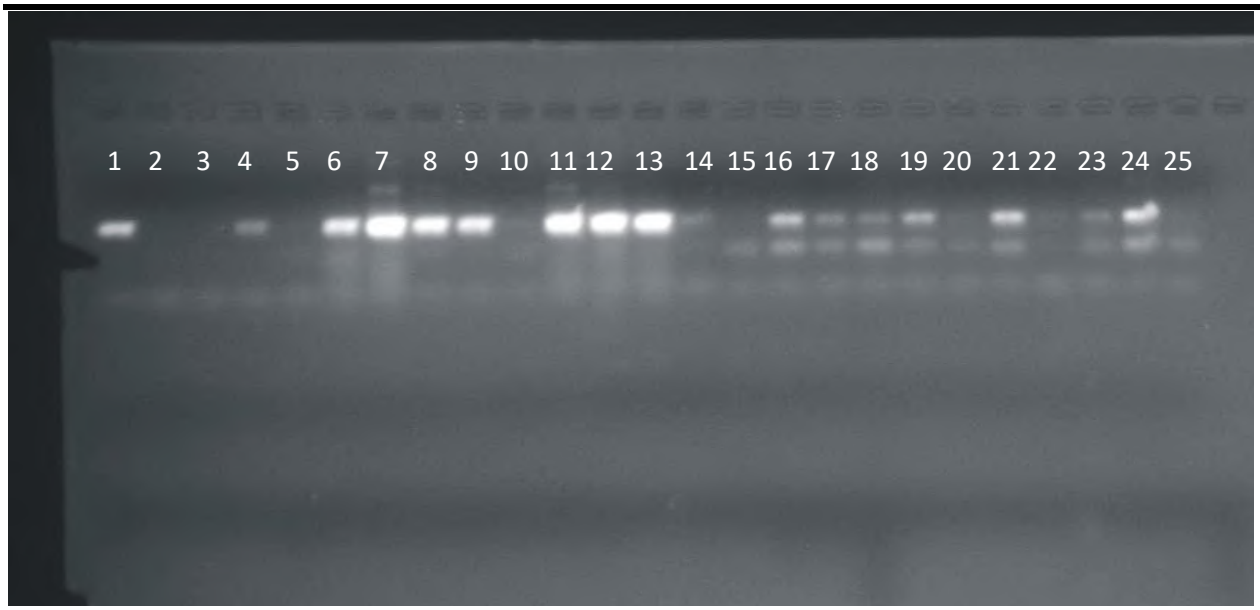

Fig. 2: Agarose gel stained with ethidium bromide showing the infection status of 23 snail samples using the Dra1 repeat.

Figure 3 represents the photo documentation of species specific of snail that are infected with *schistosomahaematobium.* This was done by the use of Restriction Fragment Length Polymorphism (RFLP) of the PCR product containing the internal transcribed spacer (ITS) of the snails using the designated restriction enzyme called Rsa 1. The enzyme is species specific and it cuts or digests each snail's DNA at required sites. The resultant DNA bands which were compared with known bands of previous restriction digest of known snail species shows that of the 23 snails, 1 was *B. globosus*, 4 were *B. truncatus* while 18 were *Physaacuta.* It was also observed from figure 1 that 23 of the 100 snails were infected and from the PCR – RFLP, as seen in figure 1 one of the snails was *B. globosus*, 4 were *B. truncatus* while 13 were *physaacuta*

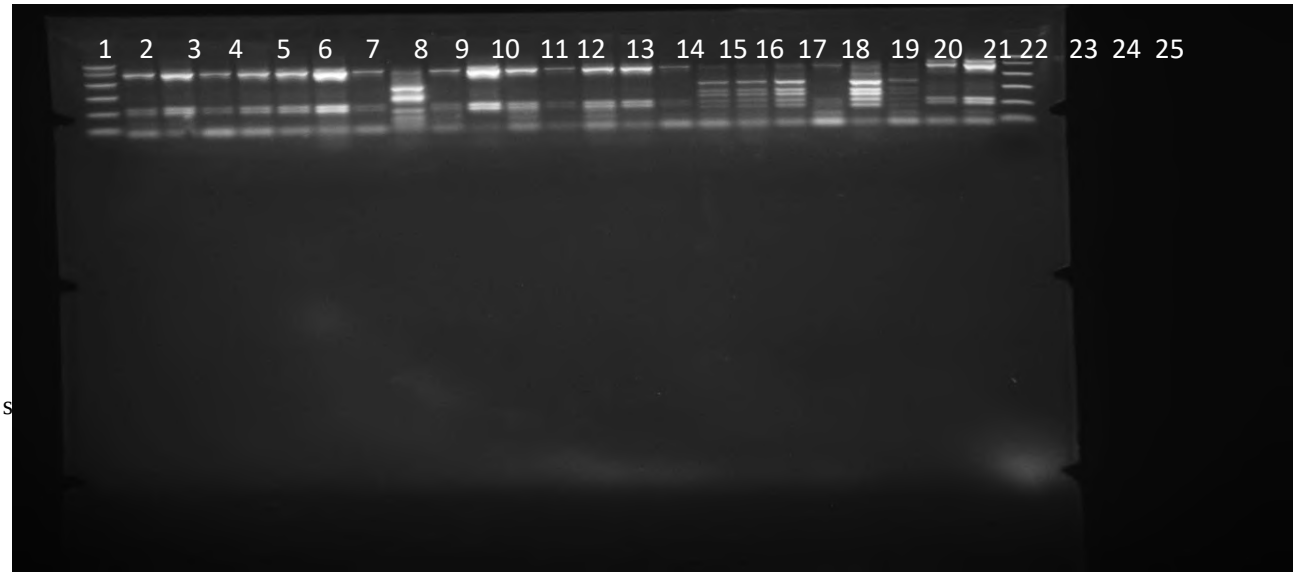

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Fig.3: The figure shows the gel analysis for confirmation by RFLP. Lane 1 and 25 are size markers, lane 2-8, 22-23 arePhysaacuta, lane 9 Bulinusglobossus, lane 17-19 and 21 are Bulinustruncatus Lanes 11, 13, 16, 20 and 22 has ambiguous profile.

Result in Table 3 showed the distribution of the *Bulinus* snail species in the communities *Bulinustruncatus* is found in Abuletitun, Iloti, Shagamu and Itori, while *B. globosus* is found in IjebuOgbere in Ijebu East Local Government respectively.

Table.3: Distributions of Schistosome Intermediate Host Species in the Communities.

Communities	**No of Snail Collected**	***Bulinus* Species**	***Physaacuta***
Abeokuta	10	0	1
Ijebu Igbo	-	-	-
AbuleTitun	10	1 (*B.truncatus*)	3
IjebuOgbere	8	1 (*B. globosus*)	2
Ipokia	8	0	0
Ado Odo/Ota	8	0	0
Obafemi Owode	9	0	0
Iloti	8	1 (*B. truncatus*)	0
Shagamu	15	1 (*B. truncatus*)	4
Itori	8	1 (*B. truncatus*)	4
Olomore	7	0	0
Ikenne	10	0	0
Total	100	5	14

IV. DISCUSSION

We observed that the snail species that may be responsible for the transmission of *S. haematobium*in these part of Ogun state are *Bulinustruncatus and Bulinusglobosus*as confirmed by the infection status revealed by PCR amplification of the *Dra1* as well as species identification by PCR-RFLP. Emejulu*et al. (*1994) reported that snails belonging to the genus *Physa*could be infected with *S. haematobium*but the snails could not carry the infection to patency. We confirmed this in our laboratory from our previous work by keeping some snails belonging to the genus *Physa*over a period of 21 days, observing them weekly for cercariae shedding. Majority of *Bulinus* snails screened belonged to the species *B. truncatus* with only one being identified as *B.globossus* from IjebuOgbere in Korede community (Ogun state). The PCR-RFLP technique of Stothard*etal.* (1996) appear to be a rapid and cost effective method of assessing Bulinusspp distribution in Nigeria and would seem the way forward for screening snails from other region of that country.

The use of sequencing of species for species determination in this study, while very effective is expensive, slow and should probably only be employed where RFLP, banding patterns are atypical or highlight the presence of cryptic species. The restrictions profiles for *B.truncatus* closely match those obtained by Stothard*et al.* (1996). Of the entire snail species collected from studied areas, only 23 were of similar morphology as the Bulinusspp (Frandsen 1983; Brown 1994). Out of these 23 snails 4 were *Bulinustruncatus*, one was *B. globossus* and 18 were of the genus physa. The factor of seasonal variation was inevitable, as fewer snails were collected during rainy seasons and more snail during the dry season. This observation agreed with that of other researchers (Agi 1995; Abdel-Nasser and Saad, 2001).

In this study morphological method was adopted in identifying the snail. This might be the problem of distinguishing Physa from Bulinusspp as both of them have almost the same appearance, except that the anterior end of Bulinusspp is blunt where that of physa is pointed. It was also observed that some of these snails had their shells fractured at the anterior end which may be due to mechanical damage. This also was a serious challenge in identifying these snails using morphological methods only. The molecular method of identification was able to overcome these challenges as it deals with DNA identification. Here the RFLP of the snails were carried out and their band patterns were compared with those of already known Bulinusspp and physaspp (which are *B. globossus, B. truncatus* and *physaacuta*).

These 23 snails were further examined for infection with *schistosomahaematobium*. Here, it was observed that even though the 23 snails were not shedding cercariae, the PCR carried out on the snail detected the presence of the Dral gene in 23 of them, the gene characteristics of *Schistosomahaematobium*. This observation shows that *Bulinustruncatus and Bulinusglobossus* are responsible for the transmission of *S. haematobium* in the study area. The observation that *B. truncatus and B. globossus are* intermediate hosts of *S. haematobium* in the studies areas agreed with those of Agi, (1995). while Okoli and Iwuala (2001), Rollinson*et al* (2001), Okafor and Ngane (2004), observed *Bulinusglobossus* in their various study sites. It was also observed that the restriction fragment analysis of

the ribosomers ITS (Internal Transcribe spacer) was a cheap and rapid method and was also found to be a promising technique for the identification of *B. truncatus*, *B. globossus*and *P. acuta*.

Out of 23 snails that were infected with *Schistosomahaematobium*, 4 were *B. truncatus*, 1 was *B. globossus* while the remains were *Physaacuta*. In IjebuOgbere the Bulinus species is *B. globossus* which is the intermediate host causing the infection in the community. In Iloti a community in Ijebu Ode, only one snail was infected with *Schistosomahaematobium* which is *B. truncatus*. Also in shagamu, ltori and Abuletitun the species of bulinus genus is *B. truncatus*

There is urgent need therefore, for the local government authority as well as that of the state government to formalise and establish feasible control programme in the area. Infection can be furthered controlled through the provision of portable water supply, eradication of snail intermediate host and health education on the relationship between water contact and infection

REFERENCES

[1] Agi, P.I. (1995) Vesical schistosomiasis at Odau village in Ahoada local government area, rivers State, Nigeria. *West African Journal of Medicine* 14: 6-10.

[2] Akogun OB, Akogun MK. 1996.Human behavior, water usage and schistosomiasis transmission in a small settlement near Yola, Nigeria. *Ann Trop Med Parasitol*1996; 90: 303-311.

[3] Brown, D. S. Freshwater Snails of Africa and Their Medical Importance. In: (2nd edition), Taylor and Francis, London 1994; pp: 609.

[4] Cowpear SG. (1973). Bilharziasis (Schistosomiasis) in Nigeria. *Ann Trop Med Parasitol*., 57: 307- 322.

[5] Driscoll AJ, Kyle JL (2005). Remais J. Development of a novel PCR assay capable of detecting a single Schistosomajaponicumcercaria recovered from Oncomelaniahupensis. Parasitol., 131: 497– 500.

[6] Ekpo UF and Mafiana, CF. (2004). Epidemiological studies of urinary schistosomiasis in Ogun State, Nigeria: Identification of high-risk communities. *Nigerian Journal of Parasitology*, 25: 111-119

[7] Emejulu A.C, Alabaronye FF, Ezenwaje HMG, Okafor FC. (1994). Investigation into the Prevalence of Urinary Schistosomiasis in the Agulu Lake Area of Anambra State, Nigeria. *J Helminthol*., 68: 119-123.

[8] Hamburger J, Weil M, Turetzky T, et al, (1989). Identification of snails infected withSchistosomes by ELISA employing monoclonal antibodies: Schistosomamansoni in laboratory snails (Biomphalariaglabrata) and in field snails (Biomphalariapfeifferi) from Kenya. *Amer J Trop Med Hygiene.*,40: 613–619.

[9] Hamburger J, He N, Abbasi I et al. (2001). Polymerase chain reaction assay based on a highly repeated sequence of Schistosomahaematobium: a potential tool for monitoring schistosome-infested water. *Amer J Trop Med Hygiene*., 65: 907– 911.

[10] Hamburger J, Hoffman O, Kariuki HC, et al. (2004). Largescale, polymerase chain reaction-based surveillance of Schistosomahaematobium DNA in snails from transmission sites in coastal Kenya: a new tool for studying the dynamics of snail infection. *Amer J Trop Med Hygiene.*, 71: 765–773.

[11] Hertel J, Kedves K, Hassan AH, et al. (2004). Detection of Schsitosomamansonicercariae in plankton samples by PCR. *ActaTropica*., 91: 43–46.

[12] Kristensen, T.K. (1987). A field guide to African freshwater snails. 2nd edn. pp. 1–51. Charlottenlund, Danish Bilharziasis Laboratory.

[13] Mafe MA, StammTVon, Utizinger J, N'Goran EK. Control of urinary schistosomiasis: an investigation into the effective use of questionnaires to identify high-risk communities and individuals in Niger State, Nigeria. Journal of Tropical Medicine and International Health 2000; 5: 53–63.

[14] Okoli, C.G. &Iwuala, M.O. (2004). The prevalence, intensity and clinical signs of urinary schistosomiasis in Imo state, Nigeria. *Journal of Helminthology* 78, 337–342.

[15] Rabello A, André Pontes L, Dias-NetoE.(2002). Recent Advances in the Diagnosis of Schist soma Infection: the Detection of Parasite DNA. Memoirs de Institut Oswaldo Cruz. 97: 171–172.

[16] Stothard JR, Hughes S, Rollinson D. (1996). Variation within the ribosomal internal transcribed spacer (ITS) from freshwater snails of the genus Bulinus. *ActaTropica*. 61: 19–29.

[17] Sturrock RF, Diaw OT, Talla I, et al. (2001). Seasonality in the transmission of schistosomiasis and in populations of its snail intermediate hosts in and around a sugar irrigation scheme at Richard Toll, Senegal. Parasitol., 123: 77–89.

[18] World Health Organization (WHO). (2002). Prevention and control of schistosomiasis and soil-transmitted helminthiasis: report of a WHO expert committee. World Health Organ Technical Report Series 2002; 912: 2–3.