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DETECTION OF HYBRID *SCHISTOSOMA HAEMATOBIUM* GROUP SPECIES IN CAMEROON BY PCR-RFLP OF THE SECOND INTERNAL TRANSCRIBED SPACER (ITS-2)

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ABSTRACT

Background: Among the species of schistosomes infecting humans, *Schistosoma haematobium* is responsible for the largest number of infections in sub-Saharan Africa with an estimated 112 million people infected with this species. *Schistosoma haematobium* has been shown to be able to interbreed with its closely related sister species, resulting in hybridisation affecting the epidemiology of these parasites. *Schistosoma haematobium, Schistosoma mansoni, Schistosoma guineensis* and *Schistosoma bovis* have all been reported in Cameroon. In this study, *S. haematobium* samples from 8 villages in Cameroon were molecularly analysed to identify hybrid schistosomes. **Methods;** PCR-RFLP analyses of the second internal transcribed spacer (ITS-2) of ribosomal DNA (rDNA) were carried out on schistosomes worms which had been isolated from 8 villages; Bessoum, Ouro-Doukoudje,Djalingo-Kapsiki, Gounougou, Njombe, Penja, Loum and Mbafam.**Results:** An ITS-2 DNA fragment of 501 bp was amplified from all the isolates of *S. haematobium* group and analysed. The *TaqI* enzymatic digestion of the ITS-2 DNA fragment revealed 3 different band profiles: Profile A (typical of *S. haematobium*) which constituted of 2 bands (158 bp and 199 bp); Profile B (typical of *S. bovis* and *S. guineensis*) which constituted of 2 bands (199 bp and 230 bp); and Profile C (probably an intermediate form) which contained all 3 bands (158bp, 199bp and 230bp). One isolate from naturally infected snail (*Bulinus globosus*) collected from the rice field in Bessoum shown profile C.

Tree worms from isolates collected from Mbafam and Djalingo-Kapsiki presented profile B, characteristic of probable hybrids. All other isolates presented with profile A, corresponding to the naturally occurring *Schistosoma haematobium* species.**Conclusions:** Our data provides an indication of hybrid *S. haematobium* group occurring in Cameroon and could serve as a reminder to inform control programs. Such findings highlight the constant need for the continued monitoring of the geographic spread and emergence of such hybrid forms.

KEYWORDS: *Schistosoma, S. haematobium, S. bovis, S. guineensis,* ITS-2, PCR-RFLP, hybridisation, Cameroon.

INTRODUCTION

Schistosomiasis, also known as, bilharzia, bilharziasis, or snail fever, is a chronic debilitating disease caused by trematodes of the genus *Schistosoma* that may affect both human beings and animals and it is an important public health and veterinary problem in many tropical and subtropical areas of the world. There are four main species that parasitize humans in Africa -*Schistosoma mansoni*, *S. intercalatum*, *S.guineensis* and *S. haematobium*-, and at least another 10 that parasitize domestic ruminants, among which, *Schistosoma bovis,* which causes intestinal schistosomiasis in ruminants, especially cattle, sheep and goats; it occurs in northern, western and eastern parts of Africa, the Mediterranean region and the Middle East. [1] *S. guineensis* is known to have a historical restricted geographical range encompassing the Lower Guinea zone, including Cameroon, Equatorial Guinea, Gabon, and Sao Tomé, [2-7] and *S. intercalatum* is strictly limited to the Democratic Republic of the Congo,[2] therefore, all the papers referring to *S. intercalatum* from areas out of the Democratic Republic of the Congo will be considered as referring to *S. guineensiss.*[8]

It is estimated that Schistosomiasis is responsible for 200 000 deaths every year.[9] A further 200 million people are probably infected and 500-600 million more are exposed to infection. [10] WHO has placed schistosomiasis as the third most devastating tropical disease, following malaria and intestinal helminthiasis. *S. bovis,* closer phylogenetically to *S. haematobium.* [11] lives in the mesenteric veins of its host. De Bont & Vercruysse.[12] suggest that at least 30% of the entire cattle populations living in endemic areas are infected with schistosomes. While most infections are subclinical, heavy infections can give rise to significant pathology, leading to severe production losses.[1]

Schistosomes undergo sexual reproduction in the definitive hosts, allowing rearrangement and the perpetuation of parasite genotype diversity. The free-swimming miracidium in the case of *S. haematobium*, must infect a snail of the genus *Bulinus* that acts as the intermediate host. Once it has penetrated the snail, the miracidium produces cercariae, which are released into water and are infective to human hosts, thus completing the life cycle. Although *B. globosus., B.senegalensis, B. truncatus* and *B. Cameruneensis* are involved in the transmission of *S.haematobium* in Cameroon.[13,14] *B. truncatus* is the most widespread and probably the most important intermediate host for transmission of *S. haematobium.*[15,13] Whereas *B. forskalii* is the only intermediate host for transmission of *S. guineensis* in Cameroon.[16] For *S. bovis*, Standard field identification keys.[17-19] based on shell morphological characteristics remains the more indicated tools for taxonomic identification of the snails to species level.

Hybridization is a way for one organism to invade the genome of another; examples of limited invasions of genomes by this method are widespread and have important implications for evolutionary biology. [20] This mechanism may generate diversity through introgression of genes across species.[21] Then, hybridization can lead to disease causing organisms acquiring novel genotypes, potentially expanding their geographical range and leading to novel ecological adaptations detrimental to human populations.[22] Schistosomes are a fascinating group of digenean trematodes because they are gonochoric, and species of the same genus that are sufficiently closely related may hybridize.[8] Hybridization, with the production of generations of viable offspring, will result from heterospecific crosses between closely related species and viable hybrids have been observed experimentally to exhibit several enhanced phenotypic characteristics such as higher fecundity, faster maturation time, higher infectivity, increased pathology and the ability to infect both intermediate snail hosts of the parental species, thereby widening their intermediate host spectrum. [23, 24]

In Cameroon, it is estimated that more than 5 million people are at risk of infection with schistosomiasis, and 2 million persons are currently infected.[25] The national epidemiological survey launched in 2010 showed the occurrence of three species of schistosomes: *Schistosoma haematobium, S. mansoni and S. guineensis.*[26, 27] Urinary schistosomiasis is endemic in the northern region of Cameroon, with some foci localised in the southern part of the country. [26] In the town of Loum, *Schistosoma guineensis* is reported to have been replaced by *Schistosoma haematobium* through exclusive competition. [28] and

introgressive hybridization.[29] In a previous study using SSCP analysis, Webster and collaborators.[30] have found hybrid between *S. haematobium* and *S. guineensis* in Loum. The introduction of the culture of rice and the practice of cattle breeding created an era of sympatry for *S.haematobium* and *S. bovis* species in the northern region of Cameroon. [31, 32] Schistosomes, however, present inherent logistical sampling problems for population genetic studies, since only egg or larval stages are directly available from living hosts. However, we now also have the methodology to collect, store and genotype larval schistosome samples directly from human (or animal) infections.[33, 34] thereby avoiding the sampling, ethical and considerable logistical and temporal disadvantages and biases of laboratory passage. Recent developments in molecular biology have increased the number and availability of molecular markers for schistosomes, providing tools to investigate many areas of Schistosoma biology, such as characterizations of new species, population genetics, interactions between species and precise species identification. [35]

In 2000, Barber and collaborators.[36] developed a simple, cheep and fast molecular method based on the restriction fragment length polymorphism (RFLP) analysis of the ITS-2 rDNA region that enabled the identification of *S. haematobium* from its closely related sister species. Sequence data from[36] indicate that *Taq1* has 4 restriction sites within the 501 bp ITS2 PCR product of *S. haematobium* and only 2 restriction sites within that of *S. bovis*, resulting in large fragments of 200 and 160 bp for *S. haematobium*, and 230 and 200 bp for *S. bovis* respectively. The remaining portions of the PCR product are cut into much smaller fragments that poorly resolved on the gel for both species. A comparison of available ITS2 sequences from GenBank indicates that these ITS2- RFLP patterns do differentiate *S. haematobium* from *S. bovis*, *S. mattheei*, S*chistosoma curassoni*, and *S. guineensis;* as the latter 4 species have essentially invariant ITS2 sequences.[37] This ITS2- RFLP patterns cannot differentiate *S.bovis* from *S. mattheei*. However a single PCR of the entire ITS region would distinguish *S. mattheei* and *S. margrebowiei* from *S. haematobium* and *S. bovis.* [36] The authors in.[36] equally demonstrated that the number and location of these RE cut sites were consistent in the ITS2 sequences of *S. haematobium* and *S. bovis* worms from various parts of Africa. Thus, the RFLP patterns could comfortably differentiate these 2 species, both as adult worms, and as single or multiple cercariae. More recently, Webster and collaborators [1] developed a high-throughput one-step multiplex PCR diagnostic method on the mitochondrial DNA (mtDNA) barcoding region to detect and discriminate between *S. haematobium* and *S. bovis* in all life cycle stages (miracidia, cercariae and adults).

In this study, we used the RFLP analysis of ITS-2 rDNA gene in order to detect natural interactions among *S. haematobium* group species within 8 villages in Cameroon. Detection of hybrids in areas where *S. haematobium* group species coexist is important in order to provide accurate transmission data for epidemiological studies and for monitoring control programmes.

MATERIALS AND METHODS

Ethical considerations

The study was approved by the National Ethics Committee of Cameroon (reference no. 072/CNE/DNM08) and was a public health intervention conducted by the Ministry of Health and the Ministry of Education. Stool and urine samples were collected from children in schools with the approval of the administrative authorities, school inspectors, directors and teachers. The objectives of the study were explained to the schoolchildren and to their parents or guardians from whom written informed consent was obtained. Children willing to participate were registered. Each child was assigned a unique identification number and results were entered in a database and kept confidential. All children who participated in the study were treated with PZQ at a dose of 40 mg/kg. Other children were treated during the mass drug administration campaign implemented by the National Programme for the Control of Schistosomiasis and Intestinal Helminthiasis (NPCSIH).

Sample collection and origin of parasite material

As part of a research project of the National Schistosomiasis Control Program, parasitological surveys were conducted in 8 villages (Bessoum, Ourodoukoudje, Djalingo-Kapsiki, Gounougou, Njombe, Penja, Loum and Mbafam) across Cameroon in 2008 (**Figure 1**).

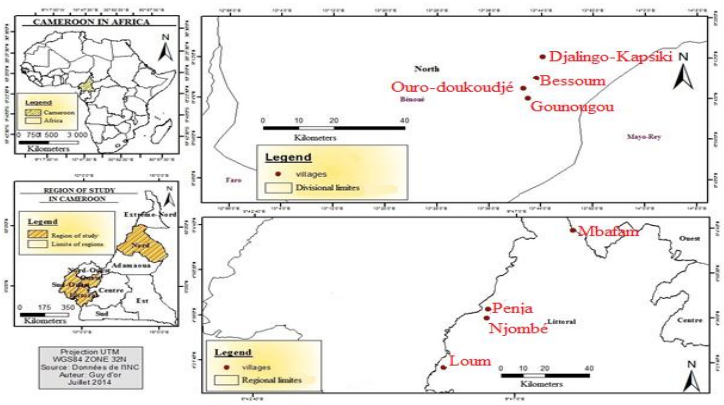

Fig. 1: Map of Cameroon showing the geographic origin of the Schistosoma isolates analysed in this study.

***Legend**: Samples were collected from 3 sites in the Littoral region (Penja, Njombe and Loum), one site in the West region (Mbafam), and four sites in the North region (Bessoum, Ourodoukoudjé, Djalingo-Kapsiki, Gounougou).*

GPS coordinates: Bessoum = Alt:194 m 9°7'57''N 13°42'50''E, OURO-Doukoudjé Alt 202m 9°6'2''N 13°41'20''E, Gounougou Alt 199m 9°4'27''N 13°41'50''E, Djalingo-Kapsiki=Alt 216 m 9°12'27''N 13°44'3''E, Njombé=Alt:77 m 4°35'39''N 9°39'58''E, Penja=Alt:137m 4°38'27''N 9°40'41''E, Loum=Alt:242m 4°19'65''N 9°20'75''E, Mbafam=Alt:734 5°2'98''N 10°00'62''E

***Schistosoma Haematobium* Collection**

The study population comprised of school children aged 6–15 years old. Two children from each of the 8 schools were recruited for the study and a total of 59 parasites infrapopulations (here defined as adult schistosomes obtained from a single child). Miracidia were hatched from eggs obtained from individual urine samples by sedimentation according to DBL (Danish Bilharziasis Laboratory) standard methods,[38] and laboratory bred 3-5mm *B.truncatus* snails, originating from Barombi Kotto Lake in the South West Region of Cameroon, were exposed individually to 5 miracidia in approximately 3 ml of spring water for 12 h. For each child, 4 to 10 snails were exposed to miracidia. The snails were maintained in the laboratory as described by DBL standard methods.[38] When the snails reached their patency date (i.e 4 weeks) they were kept in darkness for 24 h and then exposed in individual pots of 25ml fresh spring water to light room 10 a.m. to 2 p.m. to stimulate shedding. Each snail was then examined individually under a dissecting microscope for shedding of cercariae. Mice were infected individually by allowing the animals to paddle freely for 2 h in 150 ml of water containing approximately 150 (+/- 20) cercariae. The number of snails and mice used are indicated in **table 1**. Interest in obtaining adult worms rather than maintaining the cycle in the laboratory allowed the use of mice for passage of *Schistosoma haematobium* instead of hamsters. The best time for recovery of adult worms is approximately 3 ½ – 4 months after cercarial exposure.[39] This amount of time allows for recovery of fully mature worms and for maximum egg recovery from the tissues. Here, the animals were kept for at least 8 weeks in order to allow the schistosomes to mature into adults. Following this period, the animals were culled using a lethal dose of pentobarbital and the adult schistosomes were recovered using a modified vascular hepatic portal perfusion technique.[40] Euthanised animals were placed in a large Petri dish, opened dorsally and the heart exposed; care was

taken not to disturb major blood vessels. A small incision was made in the hepatic-portal vein slightly proximal to the point of mesenteric veins radiation, and 100 ml of titrated saline solution (0.85 % sodium titrate/0.85% sodium chloride) pumped under constant pressure into the left cardiac ventricle using a 21-gaugeneedle and a 10 ml syringe, were collected. The animal was then thoroughly washed in a large beaker of saline solution (85% sodium chloride), the perfusate in the Petri dish irrigated into the beaker, and the mixture allowed to stand for 10 min. The sedimented perfusate was carefully decanted. When the mesenteries are cleared of blood, we should examine the mesenteric vasculature (using a dissecting microscope) to locate other worms. Intact adult worms (singles and pairs) were removed and placed in 2 ml cryotubes, which were then snap-frozen in 70 % molecular grade ethanol for further analysis. One sample of *Schistosoma haematobium* from Nkounsoung previously characterized by isoelectric focusing was used as positive control.

Schistosoma Bovis

The *Schistosoma bovis* isolate, confirmed by RFLP as described below.[36] originated from naturally infected snail (*Bulinus globosus*) collected from the rice field in Bessoum. Snail collection was carried out on water irrigation chanel in April 2008. A total of 33 snails were collected and 3 of them shedding cercariae after exposure to light room. In a previous study in the northern part of Cameroon, Ngonseu and collaborators,[41] indicated that 7% (4/60) of infected *Bulinus globosus* shed *S.bovis* cercarea.

Table 1: Experimental Infestation of snails *Bulinus truncatus* from kotto

Villages	Isolates of Schistosome	Snails species	MD	NSE	NSS	NPS	NMI
Bessoum	Bes 1	Bu truncatus kotto	5	10	10	8	10
	Bes 2	Bu truncatus kotto	5	10	8	8	10
Ouro-Doukoudjé	Our 1	Bu truncatus kotto	5	5	5	5	5
	Our 2	Bu truncatus kotto	5	8	5	5	5
Gounougou	Gou 1	Bu truncatus kotto	5	8	7	7	5
	Gou 2	Bu truncatus kotto	5	8	7	7	5
Djalingo-Kapsiki	Dja1	Bu truncatus kotto	5	10	8	8	5
	Dja2	Bu truncatus kotto	5	4	1	1	5
Njombé	Njo 1	Bu truncatus kotto	5	10	9	9	10
	Njo 2	Bu truncatus kotto	5	8	8	8	5
Penja	Pen1	Bu truncatus kotto	5	8	7	7	6
	Pen2	Bu truncatus kotto	5	10	6	6	5
Loum	Lou1	Bu truncatus kotto	5	8	5	5	5
	Lou2	Bu truncatus kotto	5	10	8	8	5
Mbafam	Mba1	Bu truncatus kotto	5	4	1	1	6
	Mba2	Bu truncatus kotto	5	8	8	8	5

***Legend*:** *MD=miracidial dose; NSE= number of snails exposed; NSS= number of survival snails; NPS= number of positive snails; NMI= number of mice infected*

Isolation of genomic DNA

DNA was individually extracted from 63 adult worms as described by Beltran and collaborators,[42] with slight modification. Notably, before the extraction, 2 males and 2 females adult worms (for all children except Dja 2(2 males) and Mba1 (1male)) were gently separated using forceps and dried for 1 hour in a tank containing a desiccant. Next, 20 µl of 250 mM NaOH were added to each tube. After a 15 min incubation period at 25°C, the tubes were heated at 99°C for 2 min. Then, 10µl of 250 mMHCl, 5 µl of Tris-HCl (500 mM) and 5 µl of TritonX-100 (2%) were added. A second heat shock at 99°C for 2 min was performed before products were stored at -20°C. We choose this method because of financial constraints.

DNA AMPLIFICATION

The ITS2 subunit (including most of the 5.8S gene and 40 bases of the 5' of the 28S gene) was amplified using two ''universal primers'' (Ransom Hill Bioscience Inc. Ramona, CA.) ITS-3 (5'-GCA TCG ATG AAG AACGCA GC-3') and ITS-4 (5´-TCC TCC GCT TAT TGA TAT GC-3') [33]. PCR was undertaken in 25 µl volumes consisting of 250µM of each dNTP, 2.5µL of 10xPCR buffer4 (New England Biolabs), 0.5µM of ITS-3 and 0.5µM of ITS-4 primers, 0.625 U of *Taq* DNA polymerase (New England Biolabs),18.25 µL of $DdH_2O$and 10ng/µl of template DNA.[42] Amplifications were done with the following cycling parameters: 94°C for 4 min, 30 cycles of 94°C for 1 min, 50°C for 30 sec, 72°C for 45 sec, and a final extension of 7 min at 72°Cand then held at 4°C. One percent agarose gel electrophoresis with ethidium bromide staining was used to visualize and identify ITS-2-PCR products. Positive and negative controls were run simultaneously.

Restriction Fragment Length Polymorphism (RFLP) analysis of the ITS-2 regions

The positive ITS-2 PCR products were digested with *Taq*1 (New England Biolabs). Each RFLP reaction included1 µL of *Taq*1 , 2 µL of 10x Thermopol buffer, 0.2µl of BSA (10mg/mL), 7µL of PCR product(approximately 300 ng/µL of DNA) and 9.8 µL of DdH_2O, for a total volume of 20 µL per reaction. The *Taq*1 reactions were incubated at 65°C overnight.[36] The reactions were inactivated at 80°C for 20 min. 10 µL of PCR-RFLP products were separated on 2% agarose gels, with molecular size markers of a 100-bp DNA Ladder (New England Biolabs). Fragments were sized by calculating their rate of flow in the

gel and interpolation on the standard curve of rate of flow versus logarithm of molecular weight of the ladder.

RESULTS AND DISCUSSION

Adult worms obtained through laboratory passage

Laboratory passaged adult schistosome worms were obtained from all the areas sampled. We obtained for every child approximately 10 to 100 worms male and female merged except the isolate Mba 1 which has supplies only one male worm. All the analyzed isolates was couples except isolate Dja 2 (2 males) and the isolate Mba1 (1 male). From the 3 naturally infected snails, we obtained 21 adult worms (11males and 10 females). A total number of 59 worms from 16 children were analysed in this study. Isolate BesNat consisted of 4 individuals worms (2 males and 2 females) from the naturally infected snails was also included for comparison.

PCR-RFLP analysis

An ITS-2 DNA fragment of about 501 bp was amplified from all the isolates of *S. haematobium* group and no difference in product size was observed as indicated in **Figure 2**. The *TaqI* enzymatic digestion of the ITS-2 DNA fragment revealed 3 different band profiles as shown in **Figure 3**: Profile A (typical of *S. haematobium*) which constituted of 2 bands (158 bp and 199 bp); Profile B (typical of hybrid, an intermediate form) which constituted of 3 bands (158 bp, 199 bp and 230 bp) and Profile C (typical of *S. bovis*) which contained 2 bands (199bp and 230bp). One worm from isolate Mba 1 collected from Mbafam and two worms from isolate Dja 2 collected from Djalingo-Kapsiki presented profile B, characteristic of probable hybrids (intermediate form). Four worms from isolate BesNat collected from naturally infected snail from Bessoum presented profile C, characteristic of *Schistosoma bovis* species. All other isolates presented profile A, corresponding to the naturally occurring *Schistosoma haematobium* species (**Table 2**). Please palmer rewrites this to make it different from what we have in abstract (all the informations are here).

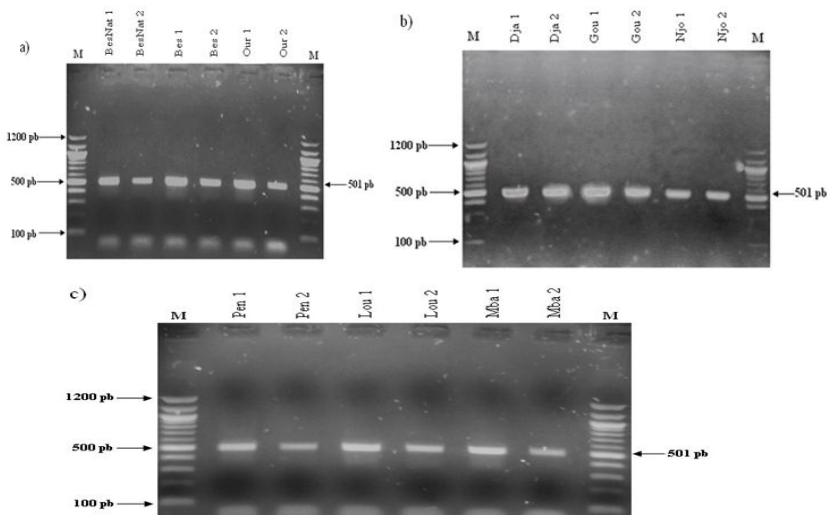

Fig. 2: ITS2-PCR Profiles obtained from the samples analysed

Legend: *Gel electrophoresis of the amplified rDNA internal transcribed spacer (ITS-2) region of Schistosoma sp strains amplified by PCR. M = 100 bp ladders.BesNat1, BestNat2, Bes1, Bes2, Our1, Our2, Dja1, Dja2, Gou1, Gou2, Njo1, Njo2, Pen1, Pen2, Lou1, Lou2, Mba1 and Mba2 are isolates from Bessoum, Ouro-doukoudjé, Djalingo-Kapsiki, Gounougou, Njombé, Penja, Loum and Mbafam respectively.*

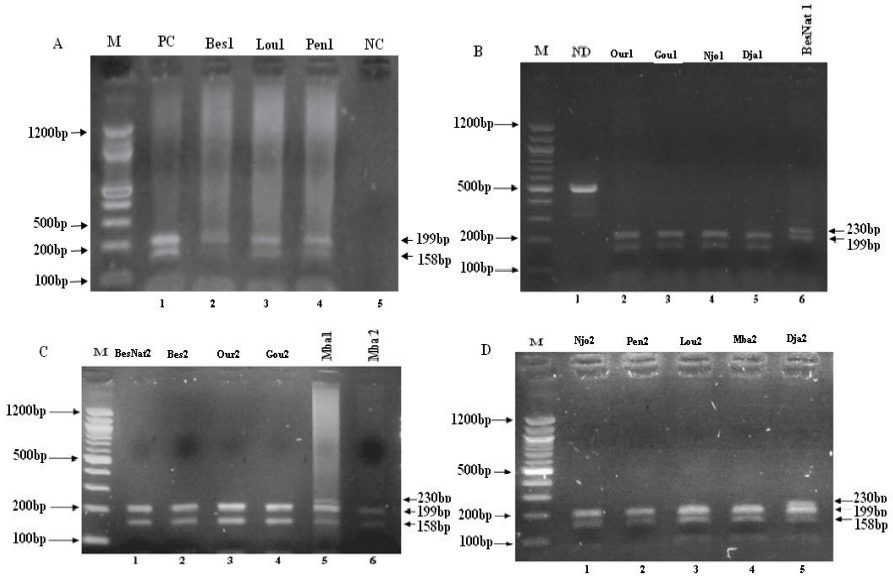

Fig.3 ITS-2 RFLP profiles (A-C) obtained from the samples analysed

Legend*: Gel electrophoresis of the amplified rDNA internal transcribed spacer (ITS-2) region of Schistosomasp strains digested with the TaqI. ND on lane B1 = Non digested, BesNat1= isolate from naturally infected snail in Bessoum rice field, PC on lane A1= Positive control (a sample from Nkounsoung previously characterized by isoelectric focusing), NC on lane A5=Negative control.M = 100 bp ladders. Bes1, Lou1, Pen1,Our1,*

Gou1,Njo1, Dja1, Mba1and Bes2, Lou2, Pen2, Our2, Gou2, Njo2, Dja2, Mba2are the first and second sets of isolates(of male adult worms) fromBessoum, Loum, Penja, Ouro-Doukoudjé, Gounougou, Njombé, Djalingo-kapsiki and Mbafam respectively. Our choice is based on the fact that male worms provided more DNA than female and consequently clearer bands following amplification.

Table 2: DNA Bands size of ribosomal gene ITS2 after digestion using *Taq I* restriction enzyme

Region	Villages	Isolate Code	individual Code for analysis	Bands size after digestion			Ribotypes
				158 bp	199 bp	230 bp	
North	Bessoum	BesNat	BesNat 1.a	0	1	1	C
			BesNat 1.b	0	1	1	C
			BesNat 2.a	0	1	1	C
			BesNat 2.b	0	1	1	C
		Bes1	Bes1.1.a	1	1	0	A
			Bes1.1.b	1	1	0	A
			Bes1.2.a	1	1	0	A
			Bes1.2.b	1	1	0	A
		Bes2	Bes2.1.a	1	1	0	A
			Bes2.1.b	1	1	0	A
			Bes2.2.a	1	1	0	A
			Bes2.2.b	1	1	0	A
	Ouro Doukoudjé	Our1	Our1.1.a	1	1	0	A
			Our1.1.b	1	1	0	A
			Our1.2.a	1	1	0	A
			Our1.2.b	1	1	0	A
		Our2	Our2.1.a	1	1	0	A
			Our2.1.b	1	1	0	A
			Our2.2.a	1	1	0	A
			Our2.2.b	1	1	0	A
	Gounougou	Gou1	Gou1.1.a	1	1	0	A
			Gou1.1.b	1	1	0	A
			Gou1.2.a	1	1	0	A
			Gou1.2.b	1	1	0	A
		Gou2	Gou2.1.a	1	1	0	A
			Gou2.1.b	1	1	0	A
			Gou2.2.a	1	1	0	A
			Gou2.2.b	1	1	0	A
	Djalingo-Kapsiki	Dja1	Dja1.1.a	1	1	0	A
			Dja1.1.b	1	1	0	A
			Dja1.2.a	1	1	0	A
			Dja1.2.b	1	1	0	A
		Dja2	Dja2.1.a	1	1	1	B
			Dja2.2.a	1	1	1	B

Table 2: Continued

Region	Villages	Isolate Code	individual Code for analysis	Bands size after digestion			Ribotypes
				158 bp	199 bp	230 bp	
Littoral	Njombé	Njo1	Njo1.1.a	1	1	0	A
			Njo1.1.b	1	1	0	A
			Njo1.2.a	1	1	0	A
			Njo1.2.b	1	1	0	A
		Njo2	Njo2.1.a	1	1	0	A
			Njo2.1.b	1	1	0	A
			Njo2.2.a	1	1	0	A
			Njo2.2.b	1	1	0	A
	Penja	Pen1	Pen1.1.a	1	1	0	A
			Pen1.1.b	1	1	0	A
			Pen1.2.a	1	1	0	A
			Pen1.2.b	1	1	0	A
		Pen2	Pen2.1.a	1	1	0	A
			Pen2.1.b	1	1	0	A
			Pen2.2.a	1	1	0	A
			Pen2.2.b	1	1	0	A
	Loum	Lou1	Lou1.1.a	1	1	0	A
			Lou1.1.b	1	1	0	A
			Lou1.2.a	1	1	0	A
			Lou1.2.b	1	1	0	A
		Lou2	Lou2.1.a	1	1	0	A
			Lou2.1.b	1	1	0	A
			Lou2.2.a	1	1	0	A
			Lou2.2.b	1	1	0	A
West	Mbafam	Mba1	Mba1.1.a	1	1	1	B
		Mba2	Mba2.1.a	1	1	0	A
			Mba2.1.b	1	1	0	A
			Mba2.2.a	1	1	0	A
			Mba2.2.b	1	1	0	A

Legend: a = Male, b = Female; 1 = Presence of DNA Fragment, 0 = Absence of DNA Fragmen

DISCUSSION

In this study, we used RFLP analysis of ITS-2 rDNA loci to identify adult schistosome worms isolated from 8 villages within 3 regions across Cameroon in 2008. The majority of the schistosomes that originated from the human urine samples presented expected *S. haematobium* ITS-2 RFLP profiles.[36] We showed for the first time how RFLP analysis of

ITS-2 rDNA loci could be used to determine the presence of possible hybrids of schistosomes in human being in Cameroon.

The RFLP technique we used demonstrated that all the samples analysed had a *S. haematobium* profile except for two isolates, which were probably hybrids and isolate BesNat which had *S.bovis* profile. Isolate Dja2 from Djalingo-Kapsiki is probably a hybrid between *S. haematobium* and *S. bovis* since that *S. guineensis* has not been reported in this northern region of Cameroon where cattle breeding is intense. [31] However, this needs to be confirmed by sequencing. Recently, Webster and collaborators.[43] and Huyse and collaborators.[44] provided conclusive molecular evidence for the natural hybridisation between *S. haematobium* and *S. bovis* and *S. haematobium* and *S. curassoni* in Senegal.

The second isolate Mba1 found in Mbafam is also probably a hybrid between *S.haematobium* and *S.guineensis.* This locality is not far from Loum where *S. haematobium* is well established,[28, 29] and follows an introgressive hybridization and competitive exclusion of the endemic *S. guineensis.*[45] There is possibly some mixing and movements of the populations between Loum and Mbafam and vice versa due to the traderoutes between Douala and Bafoussam, two major towns in Cameroon. It remains unknown whether the hybrid is from male of *S.haematobium* and female of *S.bovis* or a backcross with a parent *S. haematobium* male.

Brémond and collaborators.[46] suggested that such phenomenon is an introgression of *S.bovis* gene in female *S.hamatobium* since that there are many area where *S. haematobium* and *S.bovis* are sympatry and the hybrid are very scare.

The primers can also discriminate both species from *S. margrebowiei*, *S. leiperi* and *S. intercalatum,* as the primers will not amplify the latter three species. However, the primers do amplify *S. mattheei, S. curassoni* and *S. guineensis,* creating a double-banded profile for these three species but cannot be used to distinguish between them. In the limited areas of Africa where these species occur in sympatry with *S. haematobium* and *S. bovis*, further validation of species identity would be required by DNA sequencing of the obtained PCR products.

The distribution of *S.haematobium* and the intensive breeding of cattle in the area where *Bulinus truncatus* are found lead us to the hypothesis of hybridization.

This finding is similar to that of Moné and collaborators.[8] who found a hybrid of *S. haematobium* and *S. guineensis* in Benin using high resolution melting (HRM) analysis of ITS-2 rDNA loci. This is also in agreement with Webster and collaborators,[14] who, using a single strand conformational polymorphism, reported the presence of recombinant and hybrid forms of *S. haematobium* population in samples collected in Kumba in 1990, and Loum in 1990, 1999 and 2000.

Webster and collaborators.[1] developed a technique for the rapid diagnostic distinction based on the PCR (RD-PCR) of the mitochondrial DNA (mtDNA)of *S. haematobium* from *S. bovis*. The detection of hybrids in Cameroon using the same technique that was used to distinguish between *S. haematobium* and *S. Bovis* in Kenya, [36] speaks to its ease of use.

In this study only adult worms obtained from a single passage in mice were analysed. Indeed, previous studies have demonstrated that long-established laboratory schistosome strains may only represent 10–15% of the diversity present in that of recent field isolations, with the absence in particular of many rare alleles.[47, 48]

The methods described here could help define interactions between species such as *S. haematobium* with *S. guineensis* [49] and *S. mattheei* [24] as well as *S. bovis* and *S. curassoni* [50], especially when used with nuclear diagnostic markers described by Hamburger and collaborators,[51,52] and Barber and collaborators. [36]

Hybrids in schistosomes are known to present higher fecundity, faster maturation time and a wider intermediate host spectrum.[8] The utilisation of the 2 intermediate snail hosts will increase transmission.[43, 44] The findings presented herein underscore possible concerns of the presence of hybrids that may have an impact on disease dynamics and control strategies. The natural hybridization of *S. haematobium* and *S. guineensis* in Cameroon was reported to have important consequences on the disease dynamics. [29]

Further studies (sequencing, mtDNA, more samples, and different samples e.g. larval stages) are needed to confirm this finding. Schistosome hybrid detection requires multi-locus analysis. The ITS-2 loci need to be sequenced to confirm species and hybrids and determine if polymorphisms are shared between localities or not. The mitochondrial cytochrome c oxidase subunit I (cox1) or another mitochondrial gene needs to be amplified and sequenced to identify other hybrids or confirm hybrids since nuclear DNA will lose its hybrid signal due

to introgression.[43] Adult schistosomes cannot be directly sampled from living hosts due to their location in the blood vessels of the mesenteric system. Recent work has overcome the logistical and sampling bias problems associated with the passage through laboratory Animals and developed methods to directly genotype schistosome larvae collected from infected people. [33, 34]

This cuts out the need for the laboratory passage of the parasites, which can cause selection of certain strains. [34]

CONCLUSION

In summary the ITS-2 RFLP-PCR is a simple and cost-effective molecular tool, which can be used to detect hybridisation between certain *S. haematobium* group species, and will be extremely useful in other *S. haematobium* group epidemiological studies. Here it has been used to identify potential *S. haematobium* / *S. bovis* and / or *S. guineensis* hybrids being excreted in human urine samples. This study provides informative data to suggest that in certain areas of Cameroon *S. haematobium* is hybridising with closely related sister species, and further studies are warranted.

Competing Interests

The author(s) declare that they have no competing interests.

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