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Genetic Profiles of *Schistosoma haematobium* parasites from Malian transmission hotspot areas

Agniwo Privat^{1,2,3}, Boissier Jérôme², Sidibé Bakary¹, Dembélé Laurent¹, Diakité Assitan¹, Doumbo Safiatou Niaré², Akplogan Ahristode¹, Guindo Hassim¹, Blin Manon², Dametto Sarah², Ibikounlé Moudachirou³, Spangenberg Thomas⁴ and Dabo Abdoulaye¹

1. Department of Epidemiology of Infectious Diseases, Faculty of Pharmacy, University of Sciences, Techniques and Technologies of Bamako, IRL 3189

privatagniwo@yahoo.com (A.P); adabo@icermali.org (A.D); laurent@icermali.org (DL); thomas.spangenberg@emdgroup.com(ST); sdoumbo@icermali.org (N.S.D); bakary@icermali.org (S.B); ayassitan@yahoo.fr (D.A); banihasguindo@gmail.com (G.H); ahrisbarth1997@gmail.com (A.A.B)

2. IHPE, Univ. Montpellier, CNRS, Ifremer, Univ. Perpignan Via Domitia, Perpignan, France.

privatagniwo@yahoo.com (A.K.P); boissier@univ-perp.fr (J.B) ; manoon.blin@gmail.com (MB) ; sarah.dametto@univ-perp.fr (SD)

3. Centre de Recherche pour la lutte contre les Maladies Infectieuses Tropicales (CReMIT/TIDRC), Université d'Abomey-Calavi, Abomey-Calavi, Bénin

privatagniwo@yahoo.com (A.K.P); mibikounle2001@yahoo.fr (I.B)

4. Global Health Institute of Merck, Ares Trading S.A., a subsidiary of Merck KGaA, Darmstadt, Route de Crassier 1, 1262 Eysins, Switzerland

Corresponding author: Pr Abdoulaye Dabo, Professor of Parasitology, Malaria Research and Training Center (MRTC), IRL 3189 Environnement, Santé, Sociétés

(USTTB/UCAD/UGB/CNRST/CNRS), BP 1805 Bamako; Tél +223 76032464/63113984;

Mail: daboabdoulaye17@gmail.com

Abstract

Background - Although schistosomiasis is a public health issue in Mali, little is known about the parasite genetic profile. The purpose of this study was to analyze the genetic profile of the schistosomes of *Schistosoma haematobium* group in school aged children in various sites of Mali.

Methods - Urine samples were collected from 07 to 21 November 2021 and subjected to a filtration method for the presence *S. haematobium* eggs. The study took place in two schistosomiasis endemic villages (Fangouné Bamanan and Diakalèl), qualified as hotspots according to the World Health Organization (WHO) definition. Molecular genotyping on both Cox1 and ITS2/18S was used for eggs taxonomic assignation.

Results - A total of 970 miracidium were individually collected from 63 school aged children and stored on Whatman FTA cards for molecular analysis. After genotyping 42.0% (353/840) and 58.0% (487/840) of miracidia revealed *S. bovis* and *S. haematobium* Cox1 profiles, respectively. 95.7 (885/925) and 4.3% (40/925) revealed *S. haematobium* and *S. haematobium/S. curassoni* profiles for ITS/18S genes, respectively. There was a significant difference in the Cox1 and ITS2/18S profiles distribution according to the village ($p < 0.0001$). Overall, there was 45.6% (360/789) hybrids of which 72.0% (322/447) were from Diakalèl. Three hybrids' profiles (*Sb/Sc_ShxSc* with 2.3%); (*Sb/Sc_ShxSh* with 40.5%); (*Sh_ShxSc* with 2.8%) and one pure profile *Sh_ShxSh* with 54.4% were identified.

Conclusion - Our findings show for the first time high prevalence of hybrid schistosome in Mali. More studies are needed on population genetics of schistosomes at the human and animal

interface to evaluate the parasite's gene flow and its consequences on epidemiology of the disease as well as the transmission to humans.

Keys words: *S. haematobium*, *S. bovis*, *S. curassoni*, hybridization, Cox1, ITS/18S, Mali.

Introduction

Schistosomiasis is a parasitic disease of medical and veterinary importance that mainly affects tropical and subtropical areas. According to WHO (WHO 2022), schistosomiasis affects almost 240 million people worldwide including 85% of them living in Africa, and more than 700 million people live in endemic areas. In Mali, the national prevalence of infection estimated in 2004–2006 were 38.3% and 6.7% for *Schistosoma haematobium* (*Sh*) and *S. mansoni* (*Sm*), respectively (Clements et al. 2009). If high (*Sh*) prevalence are widespread in all Malian regions, (*Sm*) infections are mainly restricted to small clusters in central of the country (Macina and Niono districts in the Office du Niger irrigation area) and in Baguineda 30 Km from Bamako (Brinkmann et al. 1988; Clements et al. 2009; Traoré, Maude, and Bradley 1998). In addition to humans' vertebrate hosts, some *Schistosoma* species can also affect livestock. Overall, approximately 165 million parasitized animals worldwide are estimated to suffer from hemorrhagic enteritis, anemia, cachexia and most often die (De Bont and Vercruyse 1998). In Africa, three species of schistosomes are involved in livestock infestation: *S. bovis* (*Sb*) and *S. mattheei* (*Sma*) and *S. curassoni* (*Sc*) (Panzner and Boissier 2021). (*Sb*) is the most studied animal schistosome. This latter species is known to naturally infect 21 different species: 12 Bovidae, 1 Camelidae, 2 Muridae, 1 Hominidae and 3 Equidae (Panzner and Boissier 2021). (*Sb*) has been reported for first time in Mali in 1988 in the Central Niger Delta where prevalence in animal rates up to 80% (Tembely et al. 1988). Two years later prevalences of 62.5% and 85.1% for (*Sb*) and (*Sc*) were reported in the slaughterhouses of Bamako and Mopti cities

(Rollinson et al. 1990). Beyond these cases reported in Mali, (*Sb*) occurs in the Mediterranean zone and throughout sub-Saharan Africa, especially in West Africa where it has been reported in almost all countries (Burkina Faso, Gambia, Ghana, Guinea, Bissau-Guinea, Mauritania, Niger, Nigeria, Senegal, Mali, Ivory Coast, and Togo) (Kouadio et al. 2020; Moné, Mouahid, and Morand 1999)

Hybridization is a biological phenomenon that corresponds to the meeting and crossing between two distinct genetic entities previously defined as different species (Kincaid-Smith et al. 2018; Mallet 2005). Hybridization can represent a real concern in terms of parasite transmission, epidemiology and morbidity (King et al. 2015). Hybridization in parasites can complicate the prevention, effective control and treatment of the disease (Arnold 2004) because hybrid forms sometimes exhibit higher virulence and/or resistance to treatments compared to their progenitor species (King and Bertsch 2015; Taylor 1970). The study of hybridization has received renewed interest since the widespread use of molecular tools in parasite identification. Interestingly, eggs with typical (*Sb*) shape has been evidenced in human feces (Pitchford 1977), but at this date no molecular tools was available to determine the hybrid status of these eggs. Today, natural hybridizations between schistosomes have already been identified, including between different species of animal infecting schistosomes like *SbxSc* crosses in Senegal and Mali (Rollinson et al. 1990; Webster et al. 2013); between human and animal infecting schistosome species like *ShxSb* crosses in several West African countries (Angora et al. 2022; Huyse et al. 2009; Onyekwere et al. 2022; Savassi et al. 2021; Webster et al. 2013) or between human infecting schistosomes *ShxSm* crosses in Senegal and Ivory-Coast (Le Govic et al. 2019; Huyse et al. 2013).

In Mali, cases of hybridization have been reported in both animals and humans. In animal, cases of *ScxSb* hybrids were reported in cattle coming from slaughterhouses in Mopti and Bamako cities (Rollinson et al. 1990). In human, the only case of hybrid between *Sh* and

Sb was reported in ten Belgian travelers who stayed on the Dogon plateau, one of the most important endemic areas for (*Sh*) (Soentjens et al. 2016). The hybrid status of the parasite was inferred by gene sequencing of 2 eggs recovered in the stool of the patients. The nuclear ITS gene was assigned to (*Sh*) species while the Cox1 mitochondrial gene was assigned to (*Sb*) species (Cnops et al. 2013).

The present study proposes the first epidemiological study on the possible presence of schistosome hybrids of the *S. haematobium* group in hyper-endemic areas of Mali.

Methodology

Site and period of study

The study was conducted in November 2021 in two (*Sh*) endemic villages (Fangouné Bamanan and Diakalèl) in the Kayes region of western Mali. These two villages are 300 km apart. They were chosen based on their proximity to water sources (ponds in the Diéma district, the Senegal River and its tributaries in the vicinity of the city of Kayes) (Figure 1). The Kayes region is characterized by a northern Sudanese climate in the south and a Sahelian climate in the north with two main seasons: the rainy season (May-June to October) marked by average annual rainfall of up to 1000 mm in the south and 600 to 800 mm in the north, and the dry season which extends from November to April-May (Philibert et al. 2013). Agriculture and livestock are the two main economic activities of the population (Philibert et al. 2013). The Sudano-Sahelian climate of the region is indeed favorable to the cultivation of dry cereals (millet, sorghum, maize) and groundnuts, and especially to extensive livestock farming where numerous herds of cattle, sheep and goats cohabit.

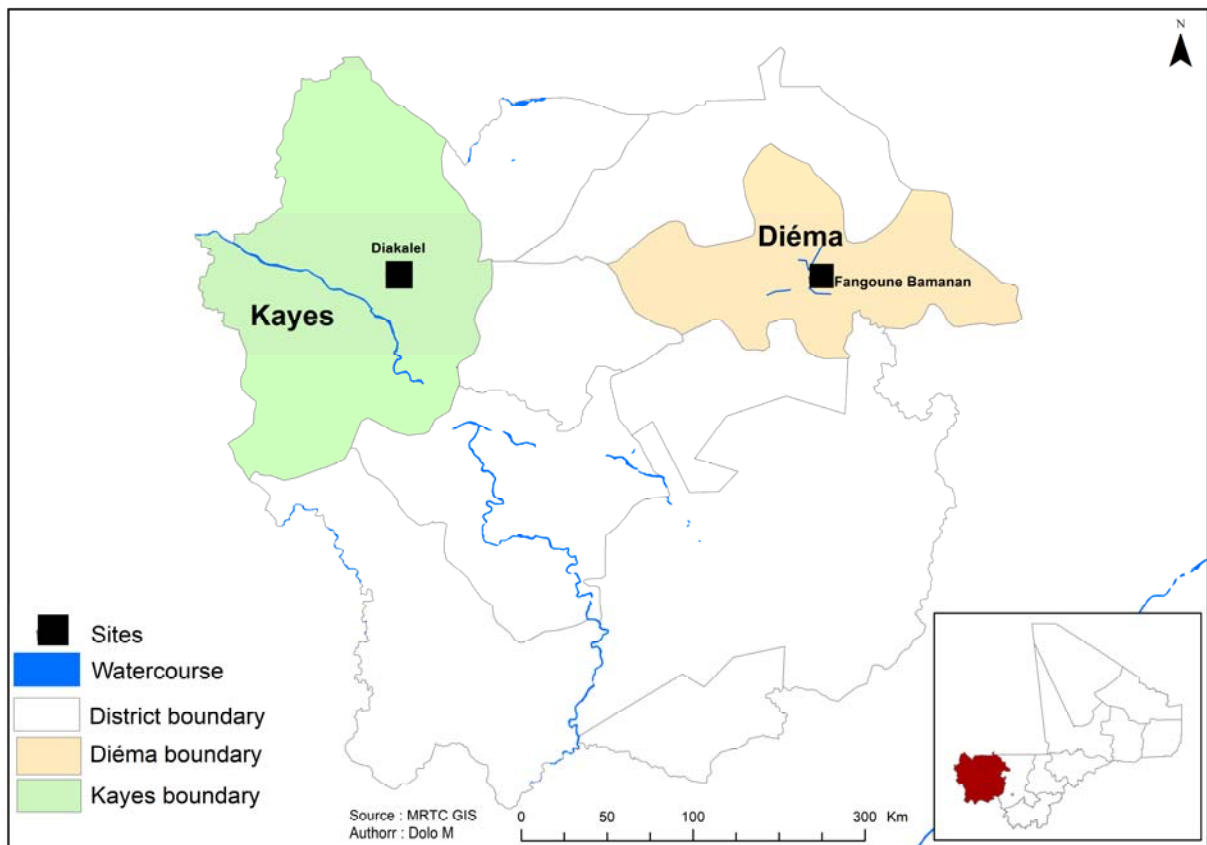


Figure 1: Kayes region (Mali, West Africa) and location of the two study sites

Parasitological methods

Urine samples were collected from 393 children (251 in Diakalèl and 142 in Fangouné Bamanan) aged 6-14 years. Each child was assigned an identification number based on the first two letters of the village name. Urine samples were collected in 60 ml sterile jars between 9 am and 2 pm. After homogenization 10 mL of urine was taken with a syringe and filtered through a numbered Whatman filter paper (catalogue CAT No. 1001-025, 25 mm) previously placed in a filter holder. The filter was then stained with 3% ninhydrin, dried and rewetted with tap water before to be read under a microscope with an X4 or X10 objective for *Sh* eggs. The number of eggs were counted and classified according to WHO (1985) recommendation as light (1-49 eggs/10mL) or heavy infection (≥ 50 eggs/10mL) (WHO 1985). Urine samples from 63 heavily infected children were used for miracidia capture on FTA cards (GE Healthcare Life Sciences; Amersham, UK). Urine samples were filtered through a 40 μ m filter (Fisherbrand™ Sterile

Cell Strainers, US, lot 100082019) and then rewashed in a watch glass using spring water. After 10 to 20 minutes contact with spring water the miracidia started to hatch. Miracidia were individually collected using a P10 micropipette set to a volume of 3 μ L and then captured on FTA card. FTA cards were stored at room temperature in a ziplock bag protected from moisture until used for molecular analysis. A total of 30 to 35 miracidia were captured per child.

Molecular analysis

Three mm² disks containing single miracidium were recovered from the FTA card using a Craft Punch and putted in a 1.5 ml Eppendorf tubes (Angora et al. 2022). The DNA was then extracted using previously published Chelex protocol (Beltran et al. 2008). A RD-PCR (Rapid Diagnostic multiplex PCR) was used to genotype the mitochondrial DNA Cox1 gene (mtDNA) (Webster et al. 2010) and ARMS (Amplification-refractory mutation system-Polymerase Chain Reaction) was used to genotype simultaneously the ITS2 and 18S nuclear genes (Blin et al. 2023). RD-PCR produces two profiles (*Sh* or *Sb/Sc*) according to the band size (size 120 pb or size 260 pb) in agarose gel. Note that the RD-PCR cannot differentiate between *Sb* and *Sc*. ARMS-PCR produces more complex profiles (4 to 6 bands) and allows to discriminate (*Sc*), (*Sb*), (*Sh*) and all hybrid combinations. RD-PCR and ARMS-PCR genotyping methods were double checked by sequencing 136 parasite profiles. The resulting sequences were assembled and manually edited using Sequencher 4.5 (Gene Codes Corporation). Sequence polymorphisms were checked and confirmed by visualizing the chromatograms of the raw sequences and then aligned with the reference sequences using MEGA software. After, the sequences were grouped by species and gene and then aligned with previously published sequences with accession numbers. For the Cox1 gene (~1200 bp), the reference sequences for (*Sh*) were (JQ397330.1 from Senegal and AY157209.1 from Mali), (*Sb*) (AJ519521.1 from Senegal and MT159594.1 from Benin) and (*Sc*) (MT579424.1 and MT579422.1 from Senegal). We compared our sequences with those of (*Sh*), (*Sb*) and (*Sc*) already published in Genbank

with the following respective accession numbers: GU257398.1, MT580950.1 and MT580946.1 for ITS (~630) and Z11976.1, AY157238.1, and AY157236.1 for 18S (~330). Species-specific SNPs allowed us to differentiate them (Blin *et al.* 2023). Combining the species assignment of the mitochondrial and nuclear genes gives the following possible genotypes: *Sb_SbxSb* ; *Sh_ShxSh* ; *Sc_ScxSc* (considered as pure) and *Sb_SbxSh* ; *Sb_SbxSc* ; *Sh_ShxSb* ; *Sh_ShxSc* ; *Sc_ScxSh* and *Sc_ScxSb* (considered as hybrid).

Statistical analysis

Gene proportions were tested using the X^2 squared or Fisher's exact tests depending on the data. The probability values (p) less than 0.05 were considered as statistically significant.

Ethical approval

The project was reviewed and approved by the Institutional Review Board (IRB) of the Faculty of Medicine, Pharmacy and Dentistry of the University of Bamako (N° 2018/71/CE/FMPOS). Community consent was obtained before the study began. Only children whose parents or guardians and themselves accepted to participate to the study were registered and requested to provide urine samples. Individuals who tested positive for schistosomiasis infection were treated with praziquantel (40 mg/kg of body weight) according to the Schistosomiasis National Control Program (PNLSH) guideline.

Results

A total of 393 urine samples were examined for *Sh* egg diagnostic (Table 1). The overall prevalence was 69.2 % (272/393). The prevalence and the intensity of infection were significantly higher in Diakalèl compared to Fangouné Bamanan ($p < 0.0001$). Urine from 63

heavy positive children (48 in Diakalèl and 15 in Fangouné Bamanan) was used to collect and store miracidia on FTA cards.

Table 1: Prevalence and intensity of *S. haematobium* in the two study sites

<i>Villages</i>	Number of children examined	Positive	Prevalence (%)	<i>p-value</i>	Negative (%)	Light infection (%)	Heavy infection (%)	<i>p-value</i>
Diakalèl	251	196	78.1	<0.0001	55 (21.9)	148 (59.0)	48 (19.1)	<0.0001
Fangouné Bamanan	142	76	53.5		66 (46.5)	61 (43.0)	15 (10.5)	
Total	393	272	69.2		121 (30.8)	209 (53.2)	63 (16.0)	

A total of 970 samples were genotyped, from which only 840 gave consistent result using the RD-PCR (Table 2 & Table S1). From these 840 samples 353 (42.0%) showed a *Sb/Sc* band size and 487 (58.0%) showed a *Sh* band size. Fifty-five samples on 353 (about 15%) randomly chosen (47 from Diakalèl and 8 from Fangouné Bamanan) with *Sb/Sc* profile where sequenced and all showed a *Sb* profile. Forty samples on 487 (8%) randomly chosen (17 from Diakalèl and 23 from Fangouné Bamanan) with *Sh* profile where sequenced and all showed a *Sh* profile. The relative repartition of each haplotype was different between Diakalèl and Fangouné Bamanan with 3 times more *Sh* haplotype in Fangouné Bamanan compared to Diakalèl. Of 970 genotyped samples, 925 gave consistent results using the ARMS-PCR, from which 885 (95.7%) showed a *ShxSh* profile and 40 (4.3%) showed a *ShxSc* profile (Table 2). Twenty-six samples on 885 (3%) randomly chosen (7 from Diakalèl and 19 from Fangouné Bamanan) with *ShxSh* profile where sequenced and all showed a *ShxSh* profile. Fifteen samples on 40 (37.5%) randomly chosen (10 from Diakalèl and 5 from Fangouné Bamanan) with *ShxSc* profile where sequenced and all showed a *ShxSc* profile.

There was a significant difference in the Cox1 profiles distribution between the two villages ($p < 0.0001$). The frequency of *Sh* ITS2/18S alleles was greater than 90% in both villages. The distribution of *ShxSh* and *ShxSc* genotypes was comparable between the two villages ($p = 0.14$).

(Table 2). Four genetic profiles were identified after genotyping miracidium using Cox1 and ITS/18S: *Sb/Sc* *cox1* x *Sh* ITS 2/*Sc*_18S (*Sb/Sc_ShxSc*); *Sb/Sc*_cox1 x *Sh*_ITS 2/ *Sh* _18S (*Sb/Sc_ShxSh*); *Sh*_cox1 x *Sh*_ITS2/*Sc* _18S (*Sh_ShxSc*) and *Sh*_cox1 x *Sh*_ITS 2/*Sh* _18S (*Sh_ShxSh*). Three hybrid profiles *Sb/Sc_ShxSc* (2.3%); *Sb/Sc_ShxSh* (40.5%); *Sh_ShxSc* (2.8%) and one pure genetic profile *Sh_ShxSh* (54.4%) have been described. Among the three hybrid profiles, the *Sb/Sc_ShxSh* profile was more widely represented in Diakalèl compared to Fangouné Bamanan (68.0% vs 4.7%). In contrast, the non-hybrid *Sh_ShxSh* genetic profile was more common in Fangouné Bamanan (88.9%) (Table 2). Overall, 45.6% (360/789) of hybrids were recorded. The highest prevalence of hybrids was observed in Diakalèl with 72.0%.

Table 2: Number and percentage (in parenthesis) of genes identified by Rapid diagnostic (RD_ PCR) for Cox 1, ARMS_ PCR for IT2/18S and parasites genetic profiles in the two *S. haematobium* parasites collected in two villages in Mali.

District	Village	<i>Haplotype Cox1</i>				<i>Génotype ITS/18S</i>									
		Total	<i>Sb/Sc</i>	<i>Sh</i>	<0.0001	Total	<i>ShxSh</i>	<i>ShxSc</i>	=0.14	Total	<i>Sb/Sc_ShxSc</i>	<i>Sb/Sc_ShxSh</i>	<i>Sh_ShxSc</i>	<i>Sh_ShxSh</i>	Total hybrides
Kayes	<i>Diakalèl</i>	475 (56.5)	333 (70.0)	142 (30.0)		526 (56.9)	508 (96.6)	18 (3.4)		447	16 (3.6)	304 (68.0)	2 (0.4)	125 (28.0)	322 (72.0)
Diéma	<i>Fangouné</i>	365 (43.5)	20 (5.5)	345 (94.5)		399 (43.1)	377 (94.5)	22 (5.5)		342	2 (0.6)	16 (4.7)	20 (5.8)	304 (88.9)	38 (11.1)
	Total	840	353 (42.0)	487 (58.0)		925	885 (95.7)	40 (4.3)		789	18 (2.3)	320 (40.5)	22 (2.8)	429 (54.4)	360 (45.6)

Discussion

We carried out parasitological and molecular study focused on collecting urine from children from a schistosomiasis-endemic area subjected to several years of mass drug treatment (MDT) with PZQ. With an average prevalence of 69.2% of *S. haematobium*, this prevalence is higher than that recently reported in 6 villages in the Kayes region (50.1%) (Agniwo et al. 2022). The prevalence and intensity were significantly higher in Diakalèl compared to Fangouné Bamanan ($p < 0.0001$). This difference between the villages could be explained by the type of watercourse, the Senegal river and its tributaries in Diakalèl more frequented than pond in Fangouné Bamanan.

The molecular analysis allowed to determine the genetic profiles on both a mitochondrial (Cox1) and two nuclear (ITS2 and 18S) genes. Of the 840 miracidia genotyped which gave consistent result using the RD-PCR in both villages, (42.0%) and (58.0%) had *Sb/Sc* and *Sh* Cox1 profiles, respectively. RD-PCR can discriminate *Sh*, *Sm* but cannot differentiate *Sb* from *Sc*. Although 15% of the randomly selected sequences were identified as *Sb* regardless of nuclear profile, we suppose that in the overall sampling, the vast majority of Cox1 came from *Sb* and not *Sc*. The percentage of *Sh* (58.0%) we found was higher than those observed in Nigeria (11%) (Onyekwere et al. 2022) or Ivory Coast (49.3%) (Angora et al. 2022) but lower than that described in Senegal (79.0%) (Boon et al. 2018) or Cameroon (95.8%) (Teukeng et al. 2022). For ITS2/18S genes, two profiles were identified with (95.7%) for *ShxSh* and (4.3%) for *ShxSc*. This percentage of *ShxSh* is higher than those observed in Nigeria (40.7%) (Onyekwere et al. 2022) or Ivory Coast (87.9%) (Angora et al. 2022) but lower than those found in Senegal (97.9%). (Boon et al. 2018) or Cameroon (92.5%) (Teukeng et al. 2022). All these studies showed that *Sh* nuclear alleles are dominant in parasite collected from humans.

Analysis of Cox1 and ITS2/18S genetic profiles was performed on 789 miracidia of which 360 (45.6%) yielded three types of hybrids: *Sb/Sc_ShxSc*; *Sb/Sc_ShxSh* and *Sh_ShxSc*. This

percentage of hybrids is lower than that observed in Ivory Coast (62.7%) (Angora et al. 2022) or Nigeria (89%) (Onyekwere et al. 2022) but higher than that observed in Cameroon (11.3%) (Teukeng et al. 2022). The hybrids with *Sb/Sc_ShxSc* profiles indicates gene flow between the different 3 schistosome species. The observation of the three-species hybrid (*Sb_ShxSc*) in our study suggests the possibility of interactions and pairing between these species as previously reported by Leger et al; (2016) in Niger. Such results confirm the potential for repeated interactions and cross-pairing among these 3 species and support the hypothesis that they are not first-generation hybrids but rather relatively recent parental and/or hybrid backcrossing (Leger and Webster 2016). The hybrid genotype profile observed in our study could be explained by the use of the same water points by human populations and animals, especially in the Sahel where water scarcity remains a recurring phenomenon. Beyond the hybridization between *Sb* and *Sh* previously shown by several authors (Angora et al. 2022; Huyse et al. 2009; Onyekwere et al. 2022; Savassi et al. 2021; Teukeng et al. 2022, Soentjens et al. 2016) we also observed *Sh_ShxSc* hybrids in children, suggesting clearly that *Sh* can hybridize naturally with *Sc*, as had also been shown in Senegal (Webster et al. 2013). The absence of samples from animals is the main limitation of this study. Genotyping of miracidia from livestock or rodents could provide information on the zoonotic transmission as was the case in Benin with cows (Savassi et al. 2020) and rodents (Savassi et al. 2021) and only with rodents in Senegal (Catalano et al. 2018). Anyway, our study can be considered a pilot study that needs to be replicated across the country to confirm or not the frequency of the 3 hybrid species *Sb_ShxSc*.

Conclusion

Our results showed for the first-time high prevalence of hybrid schistosome in human infections in the Kayes region of Mali. The presence of hybrids underscores the importance of the livestock reservoir of schistosomes therefore hampering schistosomiasis control and elimination. More studies are needed on population genetics of schistosomes at the human and

animal interface to evaluate the parasite's gene flow and its impact on epidemiology of the disease, the transmission to humans, as well as the control of the disease.

List of Abbreviations

S: Schistosoma

h: haematobium

c: curassoni

b: bovis

WHO: World Health Organization

MDA: Mass Drug Administration

FTA: Find The Agent

ITS: Internal Transcribed Spacer

ARMS: Amplification Refractory Mutation System

CAT: Catalogue

DNA: Deoxyribonucleic Acid

mt: Mitochondrial

IR: interne Reverse

IF: Interne Forward

OR: Out Reverse

OF: Out Forward

rDNA: Recombinant Deoxyribonucleic

SRB: Senegal River Basin

Ethics approval and consent to participate.

The study protocol was approved by the Institutional Ethics Committee of the Faculty of Medicine and Dentistry of Bamako under the reference number N° 2018/71/CE/FMPOS. School authorities, teachers, parents/guardians and children were informed about the objectives, procedures and potential risks and benefits of the study. Written informed consent was obtained from children's parents or legal guardians, while children provided oral assent. After sampling, a praziquantel treatment following WHO guidelines (40 mg/kg) was provided to children found with a *Schistosoma* infection. For data protection purposes, an identification number was assigned to each participant.

Consent for publication

Not applicable

Availability of data and materials

All data and materials are available in the article.

Competing interests

The authors declare that they have no conflict of interest. Ethical approval Ethical permission was obtained from the Ethic Committee of the « Faculté de Médecin et d'Odontostomatologie FMOS de Bamako, Mali. T.S. is an employee of Ares Trading S.A., an affiliate of Merck KGaA, Darmstadt, Germany.

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Authors' contributions

Conceptualization : Dabo Abdoulaye, Dembélé Laurent, Spangenberg Thomas, Boissier Jérôme, Agniwo Koba Privat , Ibikounlé Moudachirou, Niare Safiatou Doumbo

Material preparation, data collection and analysis; Agniwo Privat, Blin Manon, Dametto Sarah, Sidibe Bakary, Diakite Assitan, Niare Safiatou Doumbo, Akplogan Ahristode, Giundo Hassim, Dabo Abdoulaye, Boissier Jérôme.

Funding acquisition: Dabo Abdoulaye, Dembélé Laurent. Spangenberg Thomas.

Investigation: Dabo Abdoulaye, Agniwo Koba Privat, Sidibe Bakary, Diakite Assitan, Akplogan Ahristode Barthel

Methodology: Doumbo Safiatou Niare., Boissier Jérôme, Dabo Abdoulaye, Ibikounlé Moudachirou, Agniwo Privat, Blin Manon, Dametto Sarah,.

Project administration: Dabo Abdoulaye

Supervision: Dabo Abdoulaye, Boissier Jérôme, Niare Safiatou Doumbo, Ibikounle Moudachirou

Validation : Dabo Abdoulaye, Dembélé Laurent. Boissier Jérôme, Niare Safiatou Doumbo., Ibikounlé Moudachirou.

All authors read and approved the final manuscript.

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Compliance with ethical standards

Informed consent: The Directors of Fangouné Bamanan and Diakalel school obtained informed consent from the parents of all the schoolchildren who participated to the study. Data set availability. Sequence data will be deposited in the NCBI GenBank database Cox 1 DNA.

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