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Abstract
This study concerns the first urinary schistosomiasis case observed in Corsica (France, Europe) occurring in a 12-year-old German boy. The aim was to identify the relationship between this Schistosoma haematobium infection and other schistosomes of the Schistosoma group with terminalspined ova. Morphological and molecular analyses were conducted on the ova. The results showed that the schistosome responsible for the emergence of schistosomiasis in Corsica was due to S. haematobium introgressed by genes from S. bovis.

Keywords
Haematuria . Urinaryschistosomiasis . Corsica .France . Europe . Schistosoma . Schistosoma haematobium .Schistosoma bovis . Hybrids

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Introduction

Schistosomiasis was known to be endemic in some southEuropean areas such as southern Portugal and Greece until its eradication in the sixties of the last century (Davis 2009). Schistosomiasis had never been described to be endemic in the Mediterranean island of Corsica although Doby et al. already in 1966 had stated: "Corsica meets all the conditions required to achieve the rather unusual epidemiological chain in France, on the occasion, in summer at least: schistosome ova, Bulinus, man" (Doby et al. 1966). Almost 50 years later, the first autochthonous human schistosomiasis case acquired in Corsica was reported occurring in a 12-year-old German boy who had visited south-eastern Corsican rivers (especially Cavu river, Ste Lucie de Porto-Vecchio) in August 2013 with his family (Holtfreter et al. 2014). The case was also the first case acquired in Europe since schistosomiasis eradication in the last century. The aim of the present study was to describe the egg morphometry

of Schistosoma haematobium responsible for the boy's infection and to identify the relationships between this schistosome and species belonging to the terminal-spined egg group.

Materials and methods

Recovery of eggs and miracidia

Urine and stool samples were collected from the patient in the Tropical Medicine Unit, Heinrich-Heine-University Düsseldorf and were sent to Perpignan for further analyses. In the IHPE laboratory, University of Perpignan, the urine sample (2 l) was passed through a 25-µm pore size sieve (9‰ NaCl saline solution was used to wash the sample through the filter) and any eggs trapped on the sieve were collected in a beaker containing 20-ml saline solution. All ova were then collected with a Pasteur pipette and left to hatch in spring water at 25 °C under light for miracidial recovering. Fifteen ova were used for morphometry analyses and eight for the molecular analyses. Regarding the stool sample, the integrity of the sample was passed through a series of sieves (425, 180, 106, 45, and 25 µm pore size), washed through with 9‰ NaCl saline solution, and the residue retained on the 25-µm pore size sieve was collected in a beaker containing saline solution.

Egg morphometry analyses

The eggs were mounted individually in a 9‰ NaCl saline solution beneath cover-slips on glass slides using a Pasteur pipette. The ova were grouped according to their shapes: the round to oval eggs formed the typical S. haematobium morphotype, the more elongate and lozengical eggs formed the non-typical S. haematobium morphotype. The total length (including the terminal spine), the maximum width, and the length of the terminal spine of each egg were measured by microscopy.

Molecular analyses

DNA extraction and primers used for PCR analyses

DNA was extracted from 8 individual ova from urine, according to the protocol of Beltran et al. (2008), and was stored at −20 °C. Two adult male S. haematobium worms from Benin (West Africa), one from Sô-Tchanhoué and one from Toho, and two adult S. haematobium male worms from Gabon (Central Africa), one from Melen and one from Ekouk, were also used for DNA extraction using the EZNATissue DNA Kit (Omega Bio-tek, USA) according to the manufacturer's protocol. Partial cytochrome C oxidase (CO1) mtDNA amplification was performed by PCR using the forward primer, Cox1_schist F: 5′TCTTTRGATCATAAGCG-3′, and the reverse primer, Cox1_schist-R: 5′-TAATGCATMGGAAAAAAACA-3′
(Lockyer et al. 2003). PCR amplification of partial internal transcribed spacer-2 (ITS2) rDNAwas performed using the forward primer, 5′-GCATATCAACGCGGG-3′, and the reverse primer, 5′-ACAAACCGTAGACCGAACC-3′ (Kane et al. 2002).

CO1 mtDNA PCR

PCR was performed in a total reaction volume of 25 µl comprising 4 µl of DNA diluted tenfold for the eggs and 30 ng of DNA for the adult worms, 1× colorless GoTaq® flexi buffer (Promega, Madison, WI), 1.5 mM $MgCl_2$ (Promega, Madison, WI), 0.2 mM of each dNTP (Promega, Madison, WI), 0.4 µM forward primer, 0.4 µM reverse primer, and 1U GoTaq® G2 Hot Start Polymerase (Promega, Madison, WI). The reaction conditions

included an activation step of 95 °C for 3 min, followed by 45 cycles of 95 °C for 30 s, 48 °C for 40 s, and 72 °C for 1 min 10 s, and a final extension at 72 °C for 5 min. Among the 8 samples, 4 sequences were obtained.

ITS2 rDNA PCR

PCR was performed in a total reaction volume of 25 µl comprising 4 µl of DNA diluted tenfold for the eggs and 30 ng of DNA for the adult worms, 1× colorless GoTaq® flexi buffer (Promega, Madison, WI), 1.5 mM $MgCl_2$ (Promega, Madison, WI), 0.2 mM of each dNTP (Promega, Madison, WI), 0.4 µM forward primer, 0.4 µM reverse primer, and 1U GoTaq® G2 Hot Start Polymerase (Promega, Madison, WI). The reaction conditions included an activation step of 95 °C for 3 min, followed by 45 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 3 min. Among the 8 samples, 7 sequences were obtained.

Partial CO1 sequencing (955 bp)

Partial CO1 was sequenced (Genoscreen; Lille, France) using the reverse primer. The sequences were manually edited using Sequencher 4.5 (Gene Codes Corp.) and were aligned using the Bioedit sequence editor software version
7.0.5.3.

Partial ITS2 sequencing (154 bp)

Partial ITS2 was sequenced (Genoscreen; Lille, France) using the reverse primers. The sequences were manually edited using Sequencher 4.5 (Gene Codes Corp., Ann Arbor, USA) and were aligned using the Bioedit sequence editor software version 7.0.5.3.

Partial CO1 phylogenetic analysis

The 4 S. haematobium CO1 sequences were compared to 19 published nucleotide sequences of Schistosoma (12 sequences from S. haematobium, 3 from S. bovis, 2 from
S. guineensis, 1 from S. curassoni,and 1 from S. intercalatum) obtained at the GenBank database (Table 1) and to 4 unknown nucleotide sequences of S. haematobium from Benin and Gabon. DNA multiple sequence alignments were performed using Muscle program on the MEGA 6.06 software (Edgar 2004; Tamura et al. 2013) and refined by Gblocks 0.91b (Castresana

Country	Locality	Haplotype code	CO1 accession N°
Corsican sequences			
France (Corsica)	Ste. Lucie de Porto-Vecchio	Sh_Corsica1	KT354656
		Sh_Corsica2	KT354657
		Sh_Corsica3	KT354658
		Sh_Corsica4	KT354658
Benin sequences (Africa)			
Benin	Sô-Tchanhoué	Sh_Benin_Sô-Tchanhoué	KT354662
Benin	Toho	Sh_Benin_Toho	KT354661
Gabonese sequences (Africa)			
Gabon	Mélen	Sh_Melen	KT354660
Gabon	Ekouk	Sh_Ekouk	KT354659
S. haematobium sequences			
Egypt	area unknown	Sh_Egypt	JQ397368
Madagascar	area unknown	Sh_Madagascar	JQ397399
Mali	Niger Delta	Sh_Mali	AY157209
South Africa	Durban	Sh_South Africa	JQ397397
Cameroon	Loum	Sh_Cameroon	JQ397365
Kenya	Rekeke	Sh_Kenya	JQ397378
Gambia	area unknown	Sh_Gambia	JQ397349
Liberia	Wenshu	Sh_Liberia	JQ397350
Zambia	Simunjalala	Sh_Zambia	JQ397393
Tanzania_Zanzibar	Unguja	Sh_Zanzibar	GU257342
Sudan	Gezira	Sh_Sudan	JQ397369
Guinea Bissau	Gabu	Sh_GuineaBissau	JQ397351
S. bovis sequences			
Kenya	Nyabera Swamp, Kisumu	Sb_Kenya	FJ897160
Senegal	St Louis	Sb_Senegal	AJ519521
Tanzania	area unknown	Sb_Tanzania	AY157212
S. curassoni sequences			
Senegal	Sintiou Malem	Sc_Senegal	AJ519516
S. guineensis sequences			
	San Antonio	Sg_Sao Tome and Principe	AJ519517

Table 1 Sample and haplotype information

São Tome and Principe			
Cameroon	Edea	Sg_Cameroon	AJ519522
S. intercalatum sequences			
emocratic Republic of the Congo	Kinshasa	_Democratic Republic of the Congo	AJ519515

Sh S. haematobium, Sb S. bovis, Sc S. curassoni, Sg S. guineensis, Si S. intercalatum

2000; Dereeper et al. 2008, 2010). The probabilistic model of sequence evolution (Nei and Kumar 2000) and the gamma distribution (G) to approximate rate heterogeneity among haplotypes was performed using MEGA 6.06 software. The best model with the lowest BIC score (Bayesian Information Criterion) was HKY+G (G=0.1). The maximum likelihood method was performed using MEGA 6.06 software. Reliability for internal branch was assessed using bootstrapping procedure (1000 replicates). All bootstrap values less than 50 are not shown on the tree. The topology was rooted by S. intercalatum.

The comparison was also performed using the percentage of identity calculated from the pairwise distances data obtained by using MEGA 6.06 software.

Statistical analyses

Statistical comparison of the proportions of the two egg morphotypes was made using the Z-test on Openstat software. Means are presented with the standard error. Statistical significance was assessed at the $p<0.05$ level.

Results

Miracidial hatching

Regarding the feces sample, no ova could be recovered on the 25-µm pore size sieve. Regarding the urine sample, although no miracidia hatched from the 23 ova that could be recovered, three miracidia were seen moving inside the ova.

Egg morphometry and morphotype

Two egg morphotypes were identified on the 15 eggs that could be analyzed (Fig. 1): the first morphotype is shared by 26.7 % of the eggs and corresponds to typical round to oval eggs of the S. haematobium morphotype. The second morphotype represented 73.3 % of the eggs that are more elongate and lozengical. The proportions of each morphotype were significantly different (Z-stat=−2.56, p=0.01).

The mean length and the mean width of the eggs and the mean length of the spine of the eggs are shown in Table 2.

(CO1) mtDNA estimates of evolutionary divergence between sequences

CO1 mtDNA sequencing of the 4 Corsican samples from the urine revealed three haplotypes which displayed a maximum of 0.21 % sequence divergence. The confrontation of these sequences to databases revealed a very high identity percentage with the CO1 sequences of S. bovis (from 98.43 to 99.48 %), a high identity percentage with the CO1 sequences of S. curassoni and S. guineensis (from 93.19 to 94.97 %) and a low identity percentage with the S. haematobium sequences, including the two strains from Gabon and the strain of Benin from Toho (from 88.90 to 90.16 %).

Regarding the S. haematobium recovered from SôTchanhoué (Benin), the highest identity percentages were obtained with S. bovis and with the Corsican haplotypes (from 98.22 to 98.85 %).

Fig. 1 Egg morphology of S. haematobium from Corsica. Two egg morphotypes were observed: on the left, two non-typical S. haematobium eggs; on the right, a typical S. haematobium egg

Table 2Egg morphometry

	Mean (µm) ±standard error	Maximum	Minimum
Length	106.5±1.6	120	100
Width	42.8±1.4	55	35
Spine length	10.4±0.7	15	7
N	15	15	15

(ITS2) rDNA sequencing

The ITS2sequenceanalysisshowedthat allthesequences hada minimum of 99.98 % identity for this gene. The Corsican ITS2 sequence analysis (7 sequences) showed the presence of three different haplotypes: the first haplotype (5 sequences, accession
N° KT354663), the second (1 sequence, accession N° KT354664) and the third (1 sequence, accession N° KT354665). The first haplotype was 100 % identical to the S. haematobium sequences from Gabon (Mélen, accession N° KT354667 and Ekouk, accession N° KT354666), from Benin (Sô-Tchanhoué, accession N° KT354669), and from
Tanzania_Zanzibar, this last sequence representing the whole S. haematobium ITS2 sequences studied by Webster et al. (2013). The sequences of the Corsican haplotypes 1 and 2 harbored the specific S. haematobium SNPs (G, C, and G) at the three polymorphic positions, respectively 25, 80, and 130 (see Kane et al. 2002). The sequence of the haplotype 3 harbored oneofthethree specific “S.bovis,S.curassoniorS.guineensis” SNPs (A instead of G) at the polymorphic position 130. The Benin_Toho sequence (accession N° KT354668) harbored

the specific “S. bovis, S. curassoni or S. guineensis” SNPs (A, T, and A) at the three polymorphic positions, respectively 25, 80, and 130.

(CO1) mtDNA phylogeny

Maximum likelihood tree topology of CO1 mtDNA showed that S. haematobium from Corsica and S. haematobium from Sô-Tchanhoué in Benin do not belong to the S. haematobium group but to the S. bovis group (Fig. 2). The two strains from Gabon and the strain of Toho from Benin belong to the S. haematobium group.

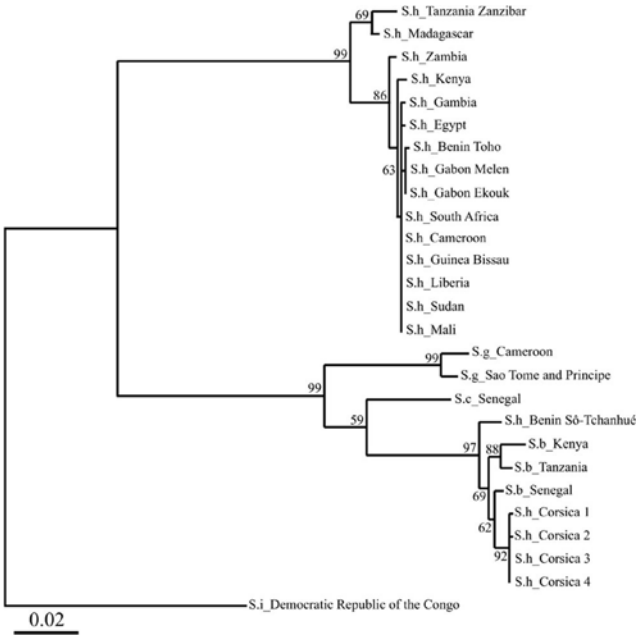

Fig. 2 Maximum likelihood tree topology showing that S. haematobium from Corsica and also from Benin-Sô-Tchanhoué belong to the S. bovis group. S.h S. haematobium, S.b S. bovis, S.c S. curassoni, S.g S. guineensis, S.i S. intercalatum. The scale shows the number of nucleotide substitutions per site

Discussion

The results obtained regarding both morphometry of the eggs and sequencing data were surprising: the schistosome responsible for the infection of the first case of schistosomiasis in Corsica (first case detected in the emergence of schistosomiasis in Europe in a German patient (Holtfreter et al. 2014)) does not correspond to a typical S. haematobium, although the boy developed a urinary schistosomiasis and terminal-spined ova could be collected only from urine and not from the feces.

Regarding the morphology of the ova from Corsica, the results showed that nearly three-quarters of the ova had an intermediate shape between typical S. haematobium eggs and typical S. bovis ova; however, these ova were small, and their mean length (107 μm) was smaller than those recorded for S. haematobium eggs (from 131 to 146 μm) (see Pitchford (1965), Loker (1983), Richard-Lenoble et al. (1993), and Moné et al. (2012)).

Regarding the sequencing data, the mitochondrial gene (COI) showed a S. bovis pattern for all the Corsican sequences, putting in evidence the existence of a mitochondrial introgressive hybridization of S. haematobium COI by S. bovis; the origin of this introgressive hybridization would come from the hybridization between a S. bovis female with a S. haematobium male and subsequent backcrosses with the parental S. haematobium. The nuclear gene (partial ITS2) showed a S. haematobium pattern for two of the three haplotypes but the sequence of

the haplotype 3 harbored a nonspecific S. haematobium SNP (A instead of G) at the polymorphic position 130. This result may be interpreted as a possible partial introgressive hybridization of S. haematobium ITS2 by S. bovis and thus a possible bidirectional introgressive hybridization.

Both morphological and molecular results showed that the emergence of schistosomiasis in Corsica was due to genetic variants of S. haematobium; these variants have been shown to have their mitochondrial genome introgressed by S. bovis. Other cases of human infections were detected in Corsica after this first caseinthe German patient (Berryetal. 2014; Boissier et al. 2015; Gautret et al. 2015); it would be interesting to analyze the status of the schistosomes in these other cases in order to know if the introduction of schistosomiasis to Corsica was a unique event or not.

A series of important questions are now to be asked: (1) Was transmission of this genetic variant only occurring during 2013 summer in Corsica or does it occur all year long? (2) Is global change responsible for this emergence in Europe since the enhance in temperaturefacilitates the cycle maintenance of the parasite? (3) Hybridization in schistosomes is known to promote heterosis and it is conceivable that this introgressed S. haematobium has a different pathogenicity towards humans. In the family of the boy, also three siblings and the father were infected but asymptomatic. Whereas the siblings had only serologic evidence of a schistosomal infection, the father excreted non-viable ova in urine.

Interesting was the case of the two strains from Benin which behaved differently. The strain from Sô-Tchanhoué behaved like the Corsican haplotypes (S. bovis COI and
S. haematobium ITS2) indicating that introgressive hybridization also occur in this zone of Africa; the origin of this introgressive hybridization would come from the hybridization between a S. bovis female with a S. haematobium male and subsequent backcrosses with the parental S. haematobium. The strain from Toho behaved originally (S. haematobium COI and non S. haematobium ITS2) indicating that nuclear introgressive hybridization of S. haematobium by genes from other schistosomesmay alsooccur in this zoneofAfrica: since the ITS2 marker only differentiates between S. haematobium and a group of three species of schistosomes (S. bovis, S. curassoni, and S. guineensis), the status of the introgressed species could not be proven; the origin of this introgressive hybridization would come from the hybridization between a
S. bovis, S. curassoni, or S. guineensis male with a S. haematobium female and subsequent backcrosses with the parental S. haematobium. This type of cross has been shown to be much less frequent (probably less viable and less successful) than the reverse cross in Northern Senegal (Huyse et al. 2009). These results show for the first time that Benin harbors cases of bidirectional introgressive hybridizations.
Similar cases were reported in Senegal between S. haematobium and S. bovis/S. curassoni (Huyse et al. 2009; Webster et al. 2013). This genetic difference between the two strains from Benin (Sô-Tchanhoué and Toho) has to be added to another difference that could be observed between these strains regarding the local adaptation towards their local intermediate snail hosts (Ibikounlé et al. 2012): while a strong local adaptation was observed with the Toho strain, no local adaptation was found with the Sô-Tchanhoué strain.

The two strains from Gabon (S. haematobium COI and S. haematobium ITS2) showed that no introgressive hybridization occurs in the Estuaire province of Gabon, confirming previous work (Mintsa Nguéma et al. 2010).

Reported natural cases of mono- or bidirectional introgressive hybridization are now more and more numerous between
S. haematobium and other species of schistosomes (S. bovis, S. guineensis, S. curassoni, S. mansoni) (Brémond et al. 1993; Tchuem Tchuenté et al. 1997; Huyse et al. 2009; Moné et al. 2012; Webster et al. 2007, 2013; this paper). The process of introgressive hybridization leads to a loss of genetic integrity of the introgressed species but, in the same time, increases the general genetic diversity of the schistosomes. Both natural selection and introgressive hybridization can lead to changing shortcuts, allowing species of schistosomes to acquire new skills very quickly rather than waiting for the implementation of the relevant genetic mutations. This should be taken into account in the epidemiological field work including diagnostic,treatment, and co-infectionanalyses (with

different species of schistosomoses or with other pathogens as Plasmodium) since hybrids may be pathogenically different from the parental species.

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Consent Written informed consent was obtained from the patient's parents for the publication of this report.

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