GENETIC VARIABILITY AMONG CERCARIAE OF THE SCHISTOSOMATIDAE (TREMATODA: DIGenea) CAUSING SWIMMER'S ITCH IN EUROPE

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Summary :

Ribosomal DNA sequences of the internal transcribed spacer 1 (ITS1) were obtained from schistosome cercariae responsible for swimmer's itch in Europe. Two types of ITS1 (1100 and 1400), which differ by the number of repeated patterns were found among cercariae shedded by *Lymnaea ovata* and *L. auricularia* (Lymnaeidae). A phylogenetic analysis of the ITS1 region showed that sequences of each type form two well-defined clades. An adult of *Trichobilharzia regenti* isolated from the nasal vessels of *Anas platyrhynchos* (Anatidae) was found to correspond to the cercaria type 1400. The sequencing of several ITS1 clones from a single cercaria of each type, as well as a specific PCR-based test suggested that both ITS1 types do not co-occur within a single individual.

KEY WORDS : cercarial dermatitis, ribosomal DNA, ITS1, size variation, *Trichobilharzia ocellata*.

Résumé : VARIABILITÉ GÉNÉTIQUE PARMI LES CERCAIRES DE SCHISTOSOMATIDAE (TREMATODA: DIGenea) RESPONSABLES DE LA DERMATITE DU BAIGNEUR EN EUROPE

Des séquences de la région ITS1 (Internal Transcribed Spacer) de l'ADN ribosomique ont été obtenues à partir de cercaires de schistosomes responsables de la dermatite cercarienne en Europe. Deux types d'ITS1 (1100 et 1400), qui diffèrent par le nombre de motifs répétés, ont été caractérisés chez des cercaires émises par *Lymnaea ovata* et *L. auricularia* (Lymnaeidae). L'analyse phylogénétique de la région ITS1 montre que les séquences appartenant à chacun des deux types forment des groupes bien définis. Un ver adulte de *Trichobilharzia regenti* trouvé dans les vaisseaux nasaux de *Anas platyrhynchos* (Anatidae) correspond au type cercarien 1400. Le séquençage de plusieurs fragments ITS1 clonés à partir d'une cercaire de chaque type, ainsi qu'un test PCR spécifique suggèrent que les deux types d'ITS1 ne sont pas présents chez un même individu.

MOTS CLÉS : dermatite cercarienne, ADN ribosomique, ITS1, variation de taille, *Trichobilharzia ocellata*.

INTRODUCTION

Cercarial dermatitis is a skin allergic response which develops following penetration of larval stages of the trematode family Schistosomatidae. It is considered as an emerging disease in Europe (de Gentile *et al.*, 1996; Chamot *et al.*, 1998). The European infection agents are represented by cercariae of bird schistosomes (Kolarova *et al.*, 1997), whose intermediate hosts are various species of aquatic snails (Dvorak *et al.*, 1999). Five genera of schistosomes parasitizing birds: *Dendritobilharzia*, *Gigantobilharzia*, *Ornithobilharzia*, *Bilharziella*, and *Trichobilharzia* have been reported in Europe (Kolarova *et al.*, 1997). The genus *Trichobilharzia* only was found in France and Switzerland (Eklu-Natey *et al.* 1985; Gay *et al.*, 1999). The

type species of this genus is *T. ocellata* (La Valette, 1855) Brumpt, 1931. The taxonomic status of *T. ocellata* is not clear and it probably represents a complex of several species (Blair & Islam, 1983; Kolarova & Horak, 1996). Recently, several *Trichobilharzia* species have been described in Europe, including *T. szidati* Neuhaus, 1952, *T. franki* Müller & Kimmig, 1994, and *T. regenti* Horak, Kolarova & Dvorak, 1998. A new *Trichobilharzia*-like cercaria species was recently described from Iceland (Kolarova *et al.*, 1999). Little is known about the natural definitive hosts of *Trichobilharzia* species; references on findings of *T. ocellata* adults in birds from Russia were presented by Sonin (1985).

Approaches classically used for schistosome species identification are morphological studies, cercarial chaetotaxy (Eklu-Natey *et al.*, 1985; Kolarova & Horak, 1996), and intermediate host specificity (Odening, 1996). However, in some cases, these approaches may not produce accurate results because the isolation of undamaged adult worms from blood vessels of birds is difficult. It seems that cercarial chaetotaxy may produce variable results, depending on the impregnation conditions of the specimens (Kock & Böckeler, 1998). As alternative methods, molecular techniques as ribosomal DNA sequencing have proved their efficiency to

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distinguish closely related schistosome species and to establish their phylogenetic relationships (Johnston *et al.*, 1993; Després *et al.*, 1995; Littlewood & Johnston, 1995; Barker & Blair, 1996; Blair *et al.*, 1997; Snyder & Loker, 2000). Similar techniques have also been used to identify the life-cycles of various digeneans by comparing adults and cercariae ribosomal DNA sequences (e.g. Cribb *et al.*, 1998; Jousson *et al.*, 1999). In the present work, we obtained ITS1 ribosomal DNA sequences of schistosome cercariae shedded by two species of *Lymnaea* snails, *L. ovata* and *L. auricularia* (Lymnaeidae) from two subalpine European lakes (lake of Geneva and lake of Annecy) which are characterised by high seasonal accumulations of aquatic birds. Sequences were also obtained from an adult worm of *Trichobilharzia regenti* isolated from the nasal vessels of *Anas platyrhynchos* (Anatidae).

MATERIAL AND METHODS

SAMPLE COLLECTION

Individuals of two species of *Lymnaea* snails (*L. ovata* and *L. auricularia*) were collected in June 2000 from several sites in the lake of Geneva (Switzerland, France) and the lake of Annecy (France). Each *Lymnaea* individual collected was isolated in a dish filled with filtered freshwater. The dishes were checked twice a day to search for spontaneous emission of schistosomatid cercariae. An adult worm preliminary identified based on microhabitat as *T. regenti* Horák, Kolárová & Dvorák 1998 was isolated from the nasal vessels of *Anas platyrhynchos* (Anatidae).

DNA EXTRACTION, PCR AMPLIFICATION, CLONING AND SEQUENCING

DNA was extracted in guanidine lysis buffer, precipitated with isopropanol and dissolved in distilled water. Only single cercariae specimens were used for DNA extraction. The ITS1 region of the ribosomal DNA was amplified using universal primers located at 3' end of the 18S rDNA (Sbr: 5'-GTAGGTGAACCTGCAGAAGG-3') and at 5' end of the 5.8S gene (5.8S1: 5'-TGTC-GATGAAGAGCGCAGC-3'). PCR amplifications were performed in a total volume of 50 µl with an amplification profile consisting of 40 cycles of 30 s at 94°C, 30 s at 52°C and 120 s at 72°C, followed by 5 min at 72°C for final extension. Amplified PCR products were purified using the High Pure PCR Purification Kit (Roche Diagnostics). Most of the PCR products were sequenced directly using amplification primers. Additionally, amplified fragments of types 1100 and 1400 were ligated in the p-GEM-T Vector (Promega) and cloned in the XL-2 Ultracompetent Cells (Stratagene).

Several colonies from each culture were sequenced in order to test the occurrence of intra-individual polymorphism. All DNA sequences were obtained using an ABI-377 automated DNA sequencer (Perkin-Elmer). A PCR test was subsequently developed to distinguish ITS1 types 1100 and 1400, using the universal reverse primer 5.8S1 and specific forward primers (spec-1100: 5'-CATTCA-TAAAGAACTGTT-3' and spec-1400: 5'-CGCAGTCAAT-CATTCAAAGT-3') designed along ITS1 sequences.

SEQUENCE ANALYSIS

The sequences were aligned manually using the program GDE 2.2 (Larsen *et al.*, 1993) and analysed using the following methods: the neighbor-joining (NJ) method (Saitou & Nei, 1987) using Kimura two-parameters distance, the maximum parsimony (MP) method, using heuristic search with the branch-swapping option included in PAUP* (Swofford, 1998) and the maximum likelihood (ML) method with a transitions-transversions ratio of 2, as implemented in the fast DNAmL program (Olsen *et al.*, 1994). The reliability of internal branches was assessed using the bootstrap method (Felsenstein, 1988). The PHYLO_WIN program (Galtier & Gouy, 1996) was used for distance computations, NJ and ML tree building and bootstrapping. DNA sequences analysed in this study have been deposited in the EMBL-GenBank database under accession numbers AJ312041-AJ312049.

RESULTS

Among 2,755 individuals of *L. ovata* and 249 individuals of *L. auricularia* collected, respectively 56 (2.03 %) and two (0.80 %) were infected by schistosomatids. Polymerase chain reaction amplification of the ITS1 rDNA region from schistosomatid cercariae gives a single product of the length 1100 or 1400 bp. Two cercariae isolated from two individuals of *L. auricularia*, as well as four cercariae isolated from four individuals of *L. ovata* generated a PCR product of 1100 bp. The adult of *T. regenti*, as well as two cercariae isolated from two individuals of *L. ovata* generated a PCR product of 1400 bp. The subsequent sequencing of amplified fragments showed that these differences are due to the presence of a variable number of repeated patterns in the ITS1. There are three kinds of subrepeats (a: 75 bp; b: 30 bp; c: 40 bp) (Fig. 1). The structure of repeated patterns of cercariae type 1100 isolated from *L. ovata* and *L. auricularia* is of type b(ab)₃. The cercariae type 1400 isolated from *L. ovata* and the adult of *T. regenti* harbour six repeat clusters of type ba-(ca)₅. The variation among repeated patterns reaches 15 nucleotide substitutions (15.1 %) within cercariae type 1100 isolated from both snail host species (Fig. 2). The variation between

Fig. 1. – Schematic representation of the ITS1 ribosomal DNA types of analysed material. *auricularia*-1100 and *ovata*-1100: 1100 bp ITS1 type of cercariae shedded by *L. auricularia* and *L. ovata*; *T. regenti* and *ovata*-1400: 1400 bp ITS1 type of an adult of *T. regenti* and a cercaria shedded by *L. ovata*. Position of universal (Sbr, 5.8S1) and specific (spec-1100, spec-1400) amplification primers are indicated. The lengths of repeated patterns are: a: 75 bp; b: 30 bp; c: 40 bp.

Fig. 2. – Alignment of ITS1 repeats within three schistosomatid cercariae (*ovata*-1400, *ovata*-1100, and *auricularia*-1100) and an adult (*T. regenti*) shown in figure 1. “-” indicates the same base as shown on upper line; “-” indicates alignment gaps. The position of repeated patterns (a, b, c) is indicated. Sequences inside boxes correspond to the pattern “b”, which is partially deleted in the first repeat of the cercaria shedded by *L. auricularia* (Au-1100_1). Ad-1400_1-6: ITS1 repeats from an adult of *T. regenti* infecting *Anas platyrhynchos*; Ov-1400_1-6: ITS1 repeats from a cercaria type 1400 shedded by *L. ovata*; Ov-1100_1-3: ITS1 repeats from a cercaria type 1100 shedded by *L. ovata*; Au-1100_1-3: ITS1 repeats from cercaria type 1100 shedded by *L. auricularia*.

repeats occurring in type 1400 reaches 24 substitutions (22.4 %) if all copies are included, and 12 substitutions (10.2 %) if the first pattern “b” is removed.

The aligned data set of ITS1 sequences comprises 1,271 sites. When all gaps are removed, there remains 811 sites, of which 89 are variable (11.0 %) and 66 informative (8.1 %). The phylogenetic analysis of ITS1 sequences using MP, NJ and ML methods shows the occurrence of two clades including all sequences of each type (Fig. 3). The sequence divergence between types 1100 and 1400 reaches 11 % (89 substitutions), whereas the within-type divergence reaches 5.6 % (47 substitutions) for the type 1100, and 1.8 % (16 substitutions) for the type 1400. The cloning of several ITS1 from single isolates showed a very low level of intraindividual variation, which does not exceed 0.6 % for the type 1100 and 1.0 %

for the type 1400. To determine if the closely related ITS1 types 1100 and 1400 could be present within a single DNA extraction, we amplified DNA from single cercaria of both types using specific primers designed in the 3' part of ITS1 (Fig. 1). We obtained positive amplifications using the specific primer spec-1100 only for the type 1100 (Fig. 4, lane 2), and primer spec-1400 only for the type 1400 (Fig. 4, lane 6). This would suggest that both types do not co-occur within a single cercaria.

DISCUSSION

The present study is the first attempt to establish the degree of genetic variability among schistosomatid cercariae causing swimmer's itch in

Fig. 3. – Phylogenetic tree of cercariae analysed in this study and one adult (*T. regenti*) inferred from ITS1 ribosomal DNA sequences by using the Maximum Parsimony (MP) method with the heuristic search option. All nodes supported by bootstrap values lower than 50 % were collapsed into a polytomy. Bootstrap values are given for MP/NJ/ML methods. Sequences belonging to each of the two types of ITS1 are shown inside boxes. Snail hosts are ov: *L. ovata*; auri: *L. auricularia*. Sequences obtained from cloned ITS1 fragments are indicated.

Europe. ITS1 ribosomal DNA sequences obtained from cercariae produced by *Lymnaea ovata* and *L. auricularia* showed the occurrence of two distinct genotypes (type 1100 and type 1400) which differ by the number of repeated patterns. In the Digenea, such a kind of ITS1 variability has been previously observed within and among individuals of *Dolichosaccus* (Luton *et al.*, 1992), *Paragonimus* (van Herwerden *et al.*, 1999) and *Schistosoma* species (Kane & Rollinson, 1994; Kane *et al.*, 1996; van Herwerden *et al.*, 1998; 1999). These authors observed the occurrence of different ITS1 variants within a single individual and suggested that it could be due to the occurrence of differences in rates of concerted evolution among repetitive ribosomal genes. We tested the hypothesis of the presence of several ITS1 types within a single cercaria by sequencing several ITS1 cloned fragments (Fig. 3), as well as by using a specific PCR test (Fig. 4). These approaches did not reveal the presence of both

types within a single individual. This would suggest that the two ITS1 types found represent distinct entities. Our data reveal that cercariae of type 1100 are parasitizing the two intermediate host species. This is in contradiction with the apparent strict intermediate host specificity of European *Trichobilharzia* species (Müller & Kimmig, 1994; Horak *et al.*, 1998). Further studies based on significant number of host and parasite samples will show if both cercariae types may occur in *L. ovata* and *L. auricularia*. There is a high genetic similarity between the cercariae of type 1400 and the adult of *T. regenti*. Further work, particularly the comparison with ITS sequences of unambiguously identified adult specimens is required to determine with more accuracy if both cercaria types really correspond to distinct species. This may help to have a better knowledge of host-parasite associations and resolve the life histories of *Trichobilharzia* species in Europe.

Fig. 4. – PCR amplifications of cercariae types 1100 (lanes 1-3) and 1400 (lanes 4-6). Primers used are: lanes 1 and 4: Sbr-5.8S1 (universal); lanes 2 and 5: specific 1100-5.8S1; lanes 3 and 6: specific 1400-5.8S1. M: Molecular weight marker.

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