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EmsB, a tandem repeated multi-loci microsatellite, new tool to investigate the genetic diversity of *Echinococcus multilocularis*

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Abstract

In order to explore the genetic diversity within *Echinococcus multilocularis* (*E. multilocularis*), the cestode responsible for the alveolar echinococcosis (AE) in humans, a microsatellite, composed of (CA) and (GA) repeats and designated EmsB, was isolated and characterized in view of its nature and potential field application. PCR-amplification with specific primers exhibited a high degree of size polymorphism between *E. multilocularis* and *Echinococcus granulosus* sheep (G1) and camel (G6) strains. Fluorescent-PCR was subsequently performed on a panel of *E. multilocularis* isolates to assess intra-species polymorphism level. EmsB provided a multi-peak profile, characterized by tandemly repeated microsatellite sequences in the *E. multilocularis* genome. This ''repetition of repeats'' feature provided to EmsB a high discriminatory power in that eight clusters, supported by bootstrap *p*-values larger than 95%, could be defined among the tested *E. multilocularis* samples. We were able to differentiate not only the Alaskan from the European samples, but also to detect different European isolate clusters. In total, 25 genotypes were defined within 37 *E. multilocularis* samples. Despite its complexity, this tandem repeated multi-loci microsatellite possesses the three important features for a molecular marker, i.e. sensitivity, repetitiveness and discriminatory power. It will permit assessing the genetic polymorphism of *E. multilocularis* and to investigate its spatial distribution in detail.

Keywords: *E. multilocularis*; Tandem repeated multi-loci microsatellite; Genetic polymorphism; Molecular ecology

1. Introduction

Echinococcus multilocularis is the causative agent of alveolar echinococcosis (AE), a mostly lethal infectious disease in humans if remaining untreated. This parasite is geographically widespread in the northern hemisphere, including Central Europe, the whole North Asian continent and North America (mainly including Canada, Alaska and a few US states) (Vuitton et al., 2003). Regional endemic areas may include peculiarities in relation to e.g. human behaviour, host immune response, host species variety, predator-prey relationship or landscape characteristics (Eckert and Deplazes, 2004). Such parameters may significantly influence prevalence in the hosts (humans, rodents and carnivores). The epidemiological heterogeneity contrasts with the genetic homogeneity of the parasite described to date (Haag et al., 1997). So far, molecular studies have exhibited very little genetic variation within *E. multilocularis* (Bowles et al., 1995; Gasser and Chilton, 1995; Okamoto et al., 1995; Bretagne et al., 1996; Haag et al., 1997; Rinder et al., 1997; Gasser et al., 1998; von Nickisch-Rosenegk et al., 1999; van Herwerden et al.,

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2000; McManus, 2006). Irrespective of the methods used (PCR-RFLP, fingerprinting or sequencing), the molecular markers (coding mitochondrial or nuclear genes, ITS or any other non-coding sequence) lacked of discriminatory power.

The precise molecular tracking of *E. multilocularis*-based upon geographically or biologically relevant markers would be helpful to support the risk management of AE. In order to increase the level of resolution or sensitivity of the molecular methods, another class of DNA markers should be explored. In this regard, DNA microsatellites, small tandemly repeated sequences (1–6 bp) widely dispersed in eukaryotic genomes, could prove to be suitable for our purposes. Their high level of polymorphism is due to the variability in the number of tandem repeats caused by slippage of DNA strands during the cellular replication process (Schlotterer, 2000). To date, microsatellite analysis seems to be a suitable approach for detecting genetic diversity in parasites (Tibayrenc, 1998; De Luca et al., 2002; Monis et al., 2002). Nevertheless, only two studies have explored the genome of *E. multilocularis* using this approach. Employing a microsatellite upstream from the coding region of the U1 snRNA gene, isolates from Europe, Japan and North America could be discriminated, but no variation was found within each of these geographic foci (Bretagne et al., 1996). More recently, two microsatellite sequences allowed to detect genetic variation among different isolates of *E. multilocularis* adults, collected on Hokkaido Island, Japan (Nakao et al., 2003). Additional polymorphic markers that could be used for assessing the detailed genetic structure of the parasite populations in relation to molecular epidemiological analyses may be helpful. The aims of the present study were (i) to characterize microsatellite sequences which are able to detect genetic variation within the genome of *E. multilocularis*, (ii) to test the reliability of the microsatellite(s) found, and (iii) to assess the relevance of the microsatellite variations in view of geno-tracking *E. multilocularis* isolates upon geographical and/or biological clustering. For this purpose, an original panel of parasites collected mainly from intermediate hosts from Switzerland, controlled by various isolates from other geographical areas, was used.

2. Materials and methods

2.1. Echinococcus isolates

The following isolates of *Echinococcus* spp. were used in this study: first, microsatellite sequences were isolated from protoscolex-DNA of G1-strain *Echinococcus granulosus* (collected from Algerian sheep). Secondly, to select the most polymorphic microsatellite sequences, a first assessment of the respective inter-specific variation was performed by using two isolates of *E. granulosus* (G1 and G6 genotypes) and one of *E. multilocularis* (Switzerland). Finally, to investigate the intra-specific variation, a total of 35 *E. multilocularis* isolates were analyzed, 27 from Europe (mainly from Switzerland), seven from Alaska, and one from Canada. All the *E. multilocularis* isolates, collected from naturally infected rodents or humans, had been subsequently maintained in laboratory rodents by serial passages every 4–6 months. To test the stability of the microsatellite in relation to the asexual metacestode multiplication, two isolates (resulting from experimental infection from two parasited French foxes), that had been continuously maintained in vivo for 1 and 7 years, respectively, by serial passage in mice every 4 months, were investigated at different sampling times. The characteristics of the *Echinococcus* isolates are summarized in Table 1.

2.2. Microsatellite isolation

2.2.1. DNA extraction

Genomic DNA was extracted from protoscoleces of *E. granulosus* (Sheep G1-strain) to avoid host DNA contamination. The High Pure PCR Template Preparation kit (Roche Diagnostics, Mannheim, Germany), based on Proteinase K digestion, was used.

2.2.2. Library construction

Approximately 1 μg of genomic DNA was digested completely with *Sau3A* (Roche). The DNA fragments were ligated into the pUC18 vector (Yanisch-Perron et al., 1985) that had been digested with *BamHI* (Roche) and dephosphorylated with alkaline phosphatase (Roche). The recombinant plasmid was inserted into *Escherichia coli* DH5α competent cells by heat shock. Transformed cells were plated on Mueller–Hinton plates containing 100 μg/ml ampicillin, 40 μg/ml X-Gal (5-bromo-4-chloro-3-indoyl-B-D-galactopyranoside) and 0.2 μl/ml IPTG (isopropyl-beta-D-thiogalactopyranoside).

2.2.3. Library screening and sequencing

Colony lifts with nylon membranes, hybridization at 45 °C in DIG Easy Hyb solution, and detection with anti-DIG antibody and colorimetric reagents NBT/BCIP were performed according to manufactures protocols (Roche Diagnostics GmbH, Mannheim, Germany). The probe was an equal mixture of digoxigenin-labelled oligonucleotides [$(TC)_{10}$, $(TG)_{10}$, $(CAC)_5CA$, $CT(CCT)_5$, $CT(ATCT)_6$ and $(TGTA)_6TG$], prepared using the DIG Oligonucleotide Tailing Kit (Roche).

2.2.4. Positive clones sequencing

Positive colonies were selected from plates and resuspended in 1 ml of distilled water. Five microlitres of this solution were used for the PCR mix containing 15 pmol of each Universal primers (M13/pUC 5′-GTAAAACGACGGCCAGT-3′ and M13/pUC reverse 5′-CAGGAAACAGCTATGAC-3′), 2.5 μl of 10X Buffer, 20 μmol of dNTP and 0.5 U of Taq polymerase in a final volume of 25 μl. PCR was performed using one step at 94 °C for 5 min, then 30 cycles with three steps at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min and a final elongation (72 °C for 5 min). PCR products were subsequently purified and automatically sequenced by using the CEQ DTCS Quick Start Master Kit (Beckman Coulter, CA, USA). For sequenced clones exhibiting more than five repeats, primers were designed from appropriate regions flanking the microsatellites, using the Primer3 software (Rozen and Skaletsky, 2000).