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Real time PCR to detect the environmental faecal contamination by *Echinococcus multilocularis* from red fox stools☆

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ABSTRACT

The oncosphere stage of *Echinococcus multilocularis* in red fox stools can lead, after ingestion, to the development of alveolar echinococcosis in the intermediate hosts, commonly small mammals and occasionally humans. Monitoring animal infection and environmental contamination is a key issue in public health surveillance. We developed a quantitative real-time PCR technique (qPCR) to detect and quantify *E. multilocularis* DNA released in fox faeces. A qPCR technique using a hydrolysis probe targeting part of the mitochondrial gene *rrnL* was assessed on (i) a reference collection of stools from 57 necropsied foxes simultaneously investigated using the segmental sedimentation and counting technique (SSCT) (29 positive for *E. multilocularis* worms and 28 negative animals for the parasite); (ii) a collection of 114 fox stools sampled in the field: two sets of 50 samples from contrasted endemic regions in France and 14 from an *E. multilocularis*-free area (Greenland). Of the negative SSCT controls, 26/28 were qPCR-negative and two were weakly positive. Of the positive SSCT foxes, 25/29 samples were found to be positive by qPCR. Of the field samples, qPCR was positive in 21/50 (42%) and 5/48 (10.4%) stools (2 samples inhibited), originating respectively from high and low endemic areas. In faeces, averages of 0.1 pg/μl of DNA in the Jura area and 0.7 pg/μl in the Saône-et-Loire area were detected. All qPCR-positive samples were confirmed by sequencing. The qPCR technique developed here allowed us to quantify environmental *E. multilocularis* contamination by fox faeces by studying the infectious agent directly. No previous study had performed this test in a one-step reaction.

1. Introduction

The parasite *Echinococcus multilocularis* is a zoonotic cestode of public health importance. Its life cycle involves carnivores (mainly the red fox *Vulpes vulpes*, in Europe, but also domestic dogs and cats) as definitive hosts. They harbour the tapeworm in their small intestines. A large range of small mammal species are involved as intermediate

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hosts, where the parasite grows in a tumor-like manner. The *E. multilocularis* eggs, i.e. the infectious agent, are released in mature proglottids into the environment with the carnivore faeces, and human or other intermediate hosts can be infected after oral ingestion. Eggs are highly resistant to environmental conditions and can remain for up to one year in the environment (Veit et al., 1995). The density of infective faeces in the field is therefore one of the critical variables to consider when estimating environmental contamination. The detection of eggs, either in fresh faeces taken from individual definitive hosts or in faeces collected in the field, is an essential challenge in monitoring environmental contamination. The eggs or part of worms (e.g. proglottid, scolex) may be present in carnivore stools sampled in the environment. Eggs can be detected after concentration by flotation with zinc chloride solution and sequential sieving (Mathis et al., 1996), and can only determine the family origin (taeniid). Species can however be identified by DNA extraction from isolated eggs followed by a specific PCR amplification (Al-Sabi et al., 2007; Deplazes and Eckert, 2001; Mathis et al., 1996; Trachsel et al., 2007). When PCRs are performed on stools, PCR inhibitors, e.g. tannic acid, calcium or humic acid (Opel et al., 2010) may prevent amplification. This problem can be overcome by using specific DNA extraction kits for stool samples and diluting the extracted DNA samples. To detect the parasite from the carnivore stools directly, Dinkel et al. (2011) recently developed an *E. multilocularis* specific real-time multiplex-nested PCR assay. In the same reaction, the carnivore host species (fox or dog) and the presence of *E. multilocularis* were identified from the total DNA extraction of the stool samples. However, it was not possible to quantify the DNA present in the initial sample in this study because of the nested amplification.

In the present study we developed a qPCR assay to detect and quantify *E. multilocularis* in fox stools from a total-DNA extraction. We analysed stools isolated after fox necropsy and stools collected in the field in contrasted endemic areas.

2. Materials and methods

2.1. DNA target and qPCR technology

For molecular diagnosis it is essential to choose a relevant marker, especially for environmental samples in which DNA can be damaged by environmental conditions. For good sensitivity, the target must be short, numerous or repeated in the genome of the organism. It should present a high interspecies polymorphism for good specificity. Mitochondria are present in large numbers in eukaryotic organisms. In *Echinococcus* species and some *Taenia* species, the complete mitochondrial genomes are now available (Nakao et al., 2007). For the present study, a target in the *ribosomal RNA* gene was chosen. The target is a part of the large ribosomal subunit gene (*rrnL*), presenting a high polymorphism in *Echinococcus* species (Nakao et al., 2007). A hydrolysis probe was chosen to increase the specificity of the PCR (Poitras and Houde, 2002). Two primers and a hydrolysis probe were designed for this PCR assay with Primer Express 3.0 on the *rrnL* gene, where an 84-bp target was defined (Fig. 1).

2.2. Copro-samples

A total of 57 intestines from autopsied foxes were first analysed for the presence of *E. multilocularis* gravid adult worms by the segmental sedimentation and counting technique (SSCT) (Umhang et al., 2011). Twenty-eight foxes were found to be *Echinococcus*-free and 29 positive for the presence of *E. multilocularis* adult worms with this technique. The worm burden was assessed from the scratched small intestines by the SSCT method, and the faeces in the colon part were sampled. Furthermore, a field sample collection of fox stools was created in January 2011. A high and a low endemic area were chosen based on a study of *E. multilocularis* prevalence in red foxes in the Jura and Saône-et-Loire areas in France (Fig. 2) (Combes et al., 2012). Between 2005 and 2010, *E. multilocularis* prevalence was estimated at 71.2% (standard error, 11.8%) in the area surrounding the town of Saint-Laurent-en-Grandvaux (5.93E, 46.58N, Jura French department), and at 1.4% (standard error 1.8%) in the area surrounding the town of Couches (4.53E, 46.90N, Saône-et-Loire French department). Fifty stool samples were randomly chosen in each area for analyses (Fig. 3). An *E. multilocularis* area with no reports of the parasite in any hosts (Jenkins et al., 2013) was also investigated in Greenland, Denmark (50 km around Ammassalik, 37.65W, 65.7N) where faeces from 14 Arctic foxes (*Vulpes lagopus*) were collected.

2.3. DNA extraction

Total DNA extraction from stool samples (colon contents and stools from the field) was performed with the QIAamp DNA Stool kit (Qiagen, Hilden, Germany), as recommended by the manufacturer: 500 mg of copro-samples were weighed, proteins were digested, DNA was bound to a QIAamp silica membrane and impurities were washed away by using an InhibitEX tablet which

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E. multi.   CT-GTGATCTTGGTGTAGTAGTTGAGATTTTTGGTCTGTTCGACCTTTTTAGCCTC--CATGAGTTGAGTTAAGACCGGCGTAAGCC
E. equinus  CT-AAGGTTTTGGTGTAGTAGTTGAGATTTTAGGTCTGTTCGACCTTTT-AGCCTT--CATGAGTTGAGTTAAGACCGGCGTGAGCC
E. ortlep.  CT-AAGGTTTTGGTGTAGTAGTTGAGATTTTAGGTCTGTTCGACCTTTTTAGCCTC--CATGAGTTGAGTTGAGACCGGCGTGAGCC
E. granu.   CT-AAGGTTTTGGTGTAGTAATTGATATTTTAGGTCTGTTCGACCTTTG-AGCCTC--CATGAGTTGAGTTAAGACCGGCGTGAGCC
E. canad.   CT-ATGGTTTTGGTGTAGTAGCTGAGATTATAGGTCTGTTCGACCATTTTAGCCTC--CATGAGTTGAGTTAAGACCGGCGTGAGCC
T. hydat.   TT-AAGGTTCAGGTGCAGCAGTTTGAACTTTTGGTCTGTTCGACCAGATTTACCTT--CATGAGTTGAGTTAAGACCGGCGTGAGCC
T. crassi.  TCTATTAATTTTATTT-GTACTTTATATACTGTGTTTAT--GACTAAAAT-ATTTTCTCGT------ACTTCTATTATTCTTTGATC
T. pisif.   TTTTA--CCTAGGTGTAGTAGTTTAGGTTTTTGGTCTGTTCGACCTTAGAAATCTC--CATGAGTTGAGTTAAGACCGGCGTGAGCC
T. taeni.   TTTAAGGCTTAGGTGTAGTAGTTTAAGTTGTTGGTCTGTTCGACCTTAAAAACCTC--CATGAGTTGAGTTAAGACCGGCGTGAGCC
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Fig. 1. Map of primers (underlined nucleotides) and probe (dotted line) on the *rrnL* gene fragment studied to specifically amplify *Echinococcus multilocularis* (AB018440), aligned with the mitochondrial referenced sequences of *E. equinus* (AF346403), *E. ortleppi* (AB235846), *E. granulosus* s.s. (AF297617), *E. canadensis* (G7) (AB235847), *Taenia hydatigena* (GC228819.1), *T. crassiceps* (AF216699.1), *T. pisiformis* (GU569096.1) and *T. taeniaeformis* (JQ663994.1).