EURO-FBP STSM: detection of *Echinococcus multilocularis* in blue berries after paramagnetic bead DNA capture.

Introduction

The flatworm *Echinococcus multilocularis,* which was ranked 3rd in a FAO risk-ranking exercise on the most important food borne parasites, is a potential health hazard to humans in several parts of Europe. The life cycle of *E. multilocularis* (figure 1) includes foxes, and other carnivores, as definitive hosts, and mainly small rodents as intermediate hosts. While the final hosts rarely develop clinical symptoms due to infection with the adult stages, the consequences for the intermediate hosts are potentially lethal. Humans can serve as intermediate hosts and become infected either from ingesting eggs after direct contact with animals shedding eggs (fox, dogs and other carnivores) or indirectly through food contaminated by *E. multilocularis* eggs. The potential for the latter mode of transmission was investigated in a recent study, where 30 % of berries from Poland was found to harbor such contamination (Lass et. al 2015). During the past decade *E. multilocularis* seem to be spreading further in Norther Europe. After it's discovery in Denmark in 2001 a surveillance program to monitor *E. multilocularis* occurrence in foxes was set in motion in Sweden and Norway. The methods used for detection available at that time was not considered adequately sensitive. Therefore, a method for detecting *E. multilocularis* in fecal samples using streptavidin coated paramagnetic bead for DNA capture with a biotinylated probe and subsequent qPCR was developed by the National Veterinary Institute of Sweden (Isaksson et al 2014). The method is reported to have higher sensitivity compared to more conventional methods (Øines et al. 2015), with one egg being the limit of detection, and it is also less laborious and time consuming. The method is currently used in the national surveillance programs for *E. multilocularis* in Norway and Sweden to screen fecal samples from foxes.

Paramagnetic bead capture of DNA relies on specific binding of a biotinylated probe to the target DNA. After hybridization the probe is coupled to streptavidin coated magnetic beads (figure 1). The bead, probe and target complexes are then separated from the sample with a magnet, effectively removing PCR inhibitors and non-target DNA that might interfere with the PCR reaction.

Currently there are no optimized methods for detecting *E. multilocularis* transmission stages in contaminated food. Unfortunately, the aforementioned method for fecal samples cannot be used directly on food, and needs to be optimized for various food matrices.

The main aims of this STSM were learn DNA-fishing techniques, with emphasis on *E. multilocularis* detection, optimize the method on berries and, depending on our progression with method development, to test the method on berries collected in Sweden.

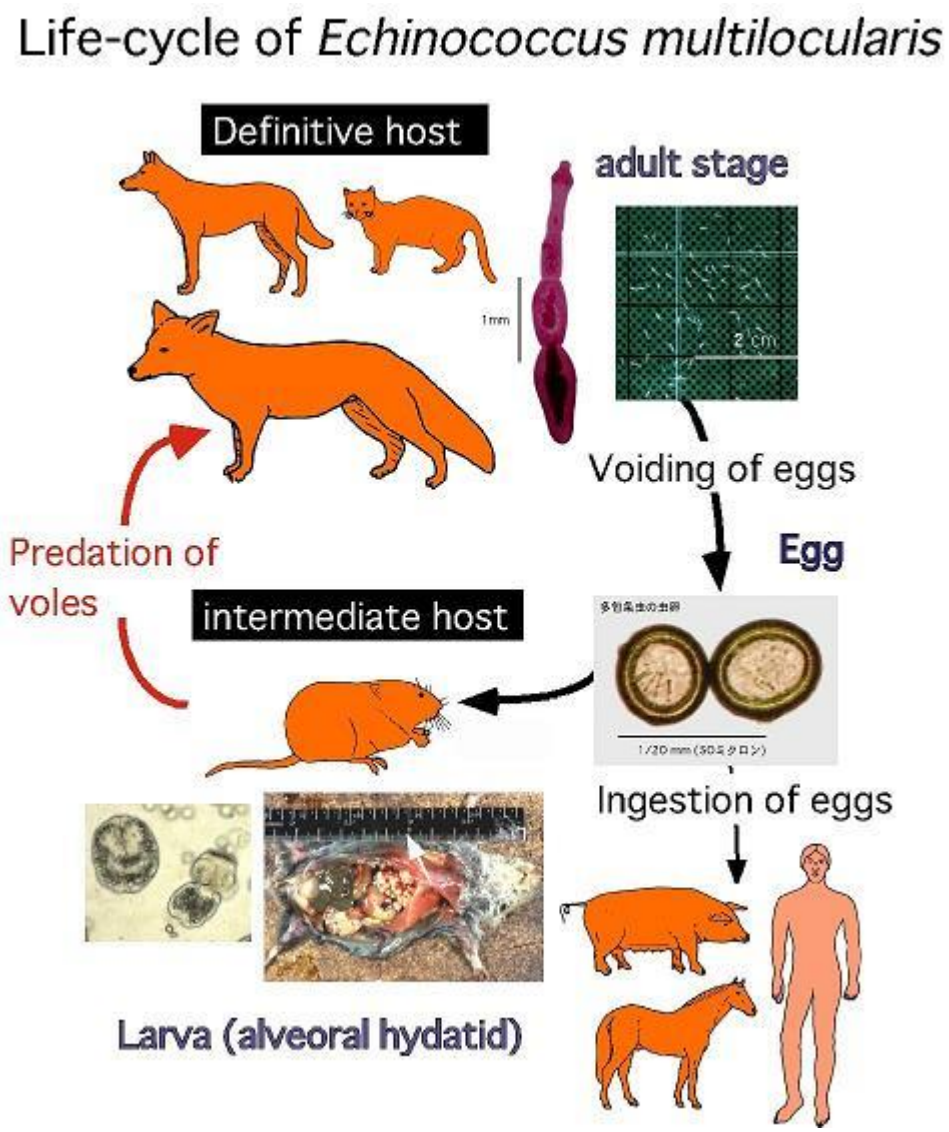

Figure 1. Life cycle of *E. multilocularis* (from Oku et al. Ecchinococcus Full-Length cDNA)

Method

The main steps for this DNA fishing method were as follows

Step 1: Homogenization

10-20 g of berries were weighed out in a 50 ml tube containing 1 ml Zirkon beads (2 mm) and 1 ml Zirkonoxid beads (0.5 mm). The tubes were then filled with lysis buffer containing Tris EDTA and SDS and 10,000 copies of a plasmid containing the *E. multilocularis* NADH dehydrogenase 1 (ND1) gene added (table 1). Alternatively, 2 ml (4.5 %) of DMSO can be added to reduce nicking effect of anthocyanins. Homogenization was performed on MP-bio homogenizer (MP-bio, figure 2), which

runs cycles of up to 60 seconds at maximum 6.5 rpm. Two different cycle setups were attempted, either 3 or 6 cycles.

Figure 2. MP-bio homogenizer. The machine can hold 2X50 ml sample tubes in each run.

Step 2 Removal of free biotin

In order to reduce the amount of free biotin in our sample, which can interfere with the streptavidin-biotin binding between the probe and paramagnetic beads, the samples were pre-treated with streptavidin-sepharose (SS) before hybridization. In short, the amount of SS needed for the number of samples was washed 3 times and re-suspended in PBS without salt and 90 µl of the SS suspension was added to each sample. Tubes were then incubated on a rotator for 20 minutes at 10 rpm before centrifugation for 15 minutes at 7,100 G and 7°C.

Step 3 Hybridization with probe

18 ml of the supernatant from step 2 was transferred to a clean centrifuge tube, to which 10 µl (1 µM) of our biotinylated capture probe was added. Tubes were then placed in a heat bath at 98°C for 15 minutes to facilitate denaturation. The annealing step was performed in a 56°C heat bath with shaker for 60 minutes. Three different capture probes developed to match regions of the (ND1) gene were used in these trials. One of the probes has previously been used to develop a protocol to extract *E. multilocularis* DNA from faecal samples (Isaksson et al 2014). This probe is complementary to a region that is approximately 1,000 base pairs away from the qPCR target sequence. In order to reduce the potential negative effect of nicking by anthocyanins, meaning that anthocyanins may cut the sequence between the capture probe site and the qPCR target, new capture probes complementary to sites closer to the qPCR target were developed.

Step 4 DNA fishing

Streptavidin coated paramagnetic beads (Dynal M-270) were prepared by washing 3 times with 0.5-1 ml and resuspension with B&W buffer in a micro-centrifuge tube placed in a magnet holder (Dynal, figure 3), after which 45 µl of bead suspension was added to each sample. The tubes were rotated for 30 minutes at 10 rpm, centrifuged for 5 minutes at 3,500 rpm and the supernatant decanted off. The pelleted beads were then re-suspended in 40 µl standard TE buffer and transferred to 1,5 ml tubes. Denaturation was performed by incubation at 99°C for 5 min before removing beads by placing tubes in a magnet holder and transferring the eluate to a 96 well PCR plate.

Figure 3. Dynal magnet holder for 1.5 ml tubes

Step 5 E. multilocularis ND1 qPCR

qPCR was performed on the eluate from step 4 according to Isaksson et al. 2014. This protocol uses a minor groove binder (MGB) hydrolysis probe (135 nM) and the primers EmMGB_F and EmMGB_R (both at 400nM) mixed with 2X Ssofast probes supermix (Bio-Rad, USA) and 4 µl of template to a final reaction volume of 15 µl. The reaction was performed on Applied Biosystems 7500 Fast instrument (Life Technologies, USA) in fast mode (ramp rate 5°C per second). The reaction was initiated by 2 minutes at 95 °C followed by 48 cycles of 95 °C for 5 seconds and 60 °C for30 seconds.

Discussion

This work was part of the preliminary development of a protocol that is intended to be used on both native and imported berries in Sweden and at the time this STSM was undertaken the protocol was still under development and we only had time to test samples spiked with plasmids. Based on the variable results and qPCR CT values obtained there is still some work left before the protocol can be used on real life samples. Working with plasmids during protocol optimization has several benefits, eg. removing risk of human infection. Although acquiring *E. multilocularis* eggs can be difficult, in order to assess the protocol sensitivity it should also be thoroughly tested on samples spiked with such eggs. When a protocol with sufficient sensitivity has been established it could be further optimized by including automated steps, eg. for bead washing, to allow faster processing of larger sample volumes.

References

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