STSM Report: Sep 13th-Sep 23rd, Norwegian University for Life Sciences (NMBU), Oslo, Norway

I would like to express my thanks to the STSM committee for granting me the funds necessary to complete this STSM and l especially want to thank the host institution (NMBU) and Professor Lucy Robertson for the opportunity to visit her laboratory and learn the methodology for the detection of *Giardia* and *Cryptosporidium* (oo)cysts in water. Also, many thanks to the whole research team, especially Kristoffer and Kjersti, for helpful instructions and patience.

Aims and objectives:

The aim of this STSM was to learn the methodology for the capture and detection of *Giardia* and *Cryptosporidium* (oo)cysts in surface water and water meant for human consumption. This methodology is directly related to my PhD thesis, as I will be investigating samples of surface and recreational waters in Serbia for the presence of *Giardia* and *Cryptosporidium* (oo)cysts. Data regarding the occurrence of these protozoa in water in Serbia is not available because the research has not been conducted to date, due mostly to the lack of expertise in sample processing and detection methodology. There are unofficial reports regarding disease outbreaks characterized by gastrointestinal symptoms, which happen mostly in the summer months in Serbia, and therefore may be attributed to infections with protozoa. However, as the capture and detection methodology for these protozoa in water is not available, water as a source of the outbreaks has not been confirmed. A number of rivers that flow through Serbia also flow through countries in the region of Southeastern Europe, in the light of which monitoring of the protozoan population, especially of those which are known to be pathogenic to humans and animals, needs to be established.

Description of work:

<u>First week</u>

The first week of the STSM was devoted to observing the procedures and techniques which were conducted. Amongst these techniques were immuno-magnetic separation (IMS), immunofluorescence staining with specific antibodies for *Cryptosporidium* and *Giardia* (IFA), PCR and gel electrophoresis.

IMS

We applied the IMS technique to process fresh coriander leaves in preparation for testing for the presence of *Giardia* cysts. For that purpose, we used commercial IMS kit for *Giardia* which contains a monoclonal antibody specific for *Giardia* cysts coupled to magnetic beads. Samples of coriander leaves were delivered to the laboratory in plastic bags at room temperature. From each bag, a total of 30g was weighed out and transferred to a stomacher bag. Next, 200ml 1M glycine buffer was added to the sample in the bag, mixed thoroughly by hand and then placed in the stomacher. The sample was processed for 1min and then the liquid phase was transferred from the plastic bag into five 50ml centrifuge tubes. The plastic bag was rinsed twice with 20ml of distilled water which was transferred into tubes and the plastic bag was discarded. The samples in the centrifuge tubes were tarred properly and centrifuged for 10min at 3000rpm. The supernatant was removed so that 5-7ml remained, the pellets were re-suspended in the remaining supernatant and the centrifugation was repeated. Next, the supernatant was again removed by aspiration, taking care that the pellet size does not exceed 0.5ml; if the volume was exceeded, the sample had to be divided into two equal parts. All pellets were vortexed and transferred into one L10 tube, which is specific for IMS. The total volume inside the L10 tube should not exceed 10ml. After the sample was prepared in this way, we continued the procedure by adding the buffers and *Giardia* specific magnetic beads from the IMS kit according to the manufacturer's instructions. For bead hybridization, the sample was incubated in a rotating mixer for at least one hour at room temperature. After this step, the sample tube was placed into a magnetic particle concentrator, MPC-1 (magnetic tube rack), which captures and attaches the beads by magnetism to the tube walls, and inverted by hand for 2min, after which the supernatant was discarded without removing the sample tube from MPC-1. Next, we removed the tube from the MPC-1, added 500µl of Q4 buffer and then transferred the whole volume into microfuge tubes. This step was repeated twice to make sure that all the beads have been collected from the walls of the L10 tube. The microfuge tube with the beads was placed into another MPC-M magnet, which can hold microfuge tubes, and inverted for 1min by hand. The supernatant was discarded by aspiration without removing the microfuge tube from MPC-M and discarded. Next, we removed the sample tube from MPC-M and added 50µl of 0.1M HCL. The sample was vortexed for 30sec twice, with a 10min pause in between vortex cycles. The addition of the acid and vortexing of the sample together promote detachment of the beads from the antibody, which remains bound to the cysts. In this way, the cysts are separated and magnetic beads can be removed from the sample, which is very

important for downstream processing—but also renders the beads non-functional and impossible to reuse with another sample. After these steps the microfuge tube was returned into MPC-M and then the liquid part of the sample, which contains the cysts, was transferred onto a glass microscope well-slide, which contained one drop of NaOH for Ph neutralization. Next, the slide was incubated at 37°C to fully dry before the sample could be fixed with methanol.

Immunofluorescence staining

Immunofluorescence staining was used for detection of *Giardia* cysts in samples we obtained after IMS. The procedure is performed using a commercially available monoclonal antibody (Mab) to *Giardia* coupled to FITC (a fluorophore which is excited at 488nm and emits light at 505nm, which is detected using appropriate filters and appears green to the viewer) according to a protocol which is used in the laboratory for parasitology at NMBU. When the sample on the microscope well-slide had completely dried after fixation, we added 50µl of a suspension containing the Mab drop-wise over the sample on the slide. The slide was then placed into a humid chamber in a thermostat set to 37°C for at least 30min. After the time elapsed, we removed the excess Mab from the slide by tapping and then added DAPI and waited for 3min. DAPI is a fluorescent dye commonly used as a counterstain to detect nucleic acids, as it is membrane permeable (under certain conditions) and can be excited at a wavelength of around 358nm (in the UV spectrum) and emits light at around 461nm, which appears blue to the viewer. The last step of the protocol was rinsing the slide with distilled water and adding one drop of mounting medium prior to cover slipping. The slide was then examined under the microscope with FITC and UV filters and found to be negative for *Giardia* cysts.

Gel electrophoresis

PCR amplicons which I previously generated using specific primers for Cryptosporidium SSU-rRNA gene were visualized by gel electrophoresis. Gel electrophoresis is a method of visualization of PCR products generated using conventional PCR techniques. The gel was made from 50ml of 1X TAE running buffer and 1g of agarose powder. To visualize the DNA on the gel, safeGreen dye was added into the gel mixture. The mixture was heated in a microwave oven to dissolve the agarose powder. Once dissolved, the gel was poured into a gel tray and the combs were placed in the appropriate slots. After approximately 30min, the agarose polymerizes and the gel tray can be transferred into a gel box where it is submerged in 1X TAE running buffer and loaded with PCR products, which are deposited into the wells generated by the combs.

After the wells were filled, a power supply was connected to the gel box set up to deliver 110V for 20min. After 20min, the gel was placed in a gel doc instrument, which passes light of the appropriate wavelength through the gel to excite the Safe-Green™ dye and makes the dye visible to the viewer by detection through appropriate emission filters, and specific amplicons were identified.

Second week

During the second week of the STSM, the major portion of the time was dedicated to the detection of Crypto/*Giardia* in water samples by IFA, while we also generated a standard curve by conventional PCR for *Cryptosporidium*.

Detection of *Cryptosporidium* and *Giardia* in water samples

Water samples which have been used for analysis were collected and delivered to the lab in 10L plastic canisters. Each sample was shaken firmly by hand prior to filtration. Water was filtered through a membrane-filter with diameter 142mm and pore size of 2 µm. The filter was washed with membrane wash buffer and distilled water carefully by hand (so that the filter membrane is not destroyed) several times during the filtration process. All the wash liquid was kept and transferred into 50ml tubes. After all the water has been filtered, the filter was submerged into a mixture of equal parts of distilled water and membrane wash buffer and washed vigorously by hand for 2min—the membrane is destroyed during this wash. The plastic container with the filter and wash liquid was transferred into a water sonicator and sonicated for 3min. This step is important because many (oo)cysts can be lost if it is skipped or not done properly. Next the filtrate is centrifuged at 1100g for 10min. The supernatant was removed up to 1cm above the pellet, to ensure that (oo)cysts are not lost. All sample pellets were combined into one 50ml centrifuge tube and distilled water was added up to 50ml and the sample was centrifuged again under the same conditions. The supernatant was discarded and the pellet was used for IMS according to the protocol described earlier—the only difference was the use of *Giardia*/ *Cryptosporidium* (G/C) combo kit, with the appropriate Mab's coupled to magnetic beads and buffers.

PCR standard curve for *Cryptosporidium* oocysts

For the detection of *Cryptosporidium* the laboratory at NMBU uses primers for SSU rRNA (nested PCR) originally developed by Xiao et al. (2001) but modified. The amplicon length is (1325bp). Seal herpes virus (PHHV) was used as an extraction control and was spiked into each sample prior to extraction. The amplicon length of PHHV is around 80bp. For the standard curve we used purified and concentrated oocysts isolated from an infected calf and stored in water at 4 C. In order to generate a standard curve, which covers a wide range of cysts, we used 2000, 200, 20 and 2 cysts. Cysts were counted using a KOVA GLASSTIC slide 10 with grids. Next step was extraction according to a standard protocol for DNA isolation of *Cryptosporidium*/*Giardia* used in the Laboratory of Parasitology of NMBU. Briefly, the protocol is a combination of freeze/thaw cycles and extraction according to the manufacturer's instructions for Qiagen's QIAamp DNA mini Kit. After gel electrophoresis, we were able to observe three out of four amplicons for *Cryptosporidium*; the amplicon corresponding to 200 cysts unfortunately was not visible. All amplicons for PHHV were present. We can conclude that the extraction worked fine, and as we used the same Mastermix for *Cryptosporidium*, it is likely that the reason we did not see an amplicon for 200 cysts is due to a technical problem.

Main results

In summary, the IFA was negative for all water samples tested (n=6). This means that no *Cryptosporidium* and *Giardia* (oo)cysts were present in a total of 120L of water. As the IFA was negative, there was no need to perform PCR.

Future collaboration possibilities

Now that I have mastered isolation, concentration and detection of *Cryptosporidium* and *Giardia* in water, the only remaining protozoa I am interested in for my PhD thesis is *Toxoplasma gondii.* Professor Robertson's laboratory has developed a technique for capturing *T. gondii* oocysts from water, which is a modification of IMS technique and it is called lectin magnetic separation (LMS). Due to time constrains and a full training schedule, I was unable to perform LMS during this STSM and hope to be able to do that during a future STSM.

Future plans

In the future I plan to collect and analyze surface water samples from different regions of Serbia for the presence of (oo)cysts of *Cryptosporidium* and *Giardia* using the methodology I have learned during this STSM. The results generated will offer insight into the level of contamination of water, while species typing by PCR will allow us to assess the biodiversity of waterborne pathogenic protozoa. This information is important for the healthcare system because it will provide information necessary for outbreak tracing and valuable to the research community.

Outputs produced

In light of the fact that this STSM was devoted to learning and training novel techniques, currently there is no output in terms of research results leading to publications. However, once I begin collecting and processing water samples in Serbia , I will get a dataset. This dataset will be published as it represents new information regarding protozoa in water in Serbia.