SHORT TERM SCIENTIFIC MISSION (STSM) – SCIENTIFIC REPORT

The STSM applicant submits this report for approval to the STSM coordinator

Action number: FA1408
STSM title: Detection of Cryptosporidium spp., Giardia spp. and T. gondii in water
STSM start and end date: 22/01/2018 to 02/02/2018
Grantee name: Vladimir Cirkovic

PURPOSE OF THE STSM/

(max.500 words)

My PhD project deals with detection of *Giardia*, *Cryptosporidium* and *Toxoplasma gondii* (oo)cysts in surface and recreational waters in Serbia. This is a novel area of research in this country, there is no data concerning this problem at all, and the methodology for this work has not yet been fully introduced. Accordingly, the purpose of this STSM was to learn new techniques and methods relevant for my PhD thesis and at the same time to further the knowledge I acquired during my previous visit to Prof. Lucy Robertson's Laboratory for Food Safety and Infection Biology, at the Norwegian University of Life Sciences (NMBU). This is actually the Norwegian national reference laboratory for detection of parasitic protozoa in water. Last September, during my first STSM, I learned techniques for the detection of *Giardia spp*. and *Cryptosporidium* spp., such as immuno-magnetic separation (IMS), immuno-fluorescence (IFA) and SSU rRNA PCR.

This STSM was devoted to learning the methods for the detection of *T. gondii* oocysts in water, specifically to learning a technique of lectin magnetic separation (LMS). LMS is the only technique known so far for the isolation of *T. gondii* oocysts from water, and it is still in development. Unlike IMS, which is done using a commercial kit for *Cryptosporidium* and *Giardia*, LMS is quite elaborate due to the nature of *T. gondii* itself. The oocyst wall of *T. gondii* is unstable and changes with ageing, which is not the case with (oo)cysts of *Cryptosporidium* and *Giardia*. LMS is currently a technique available only in specialized laboratories as it requires manual preparation of reagents and materials.

So during this STSM I got acquainted with methods for priming of *T. gondii* oocyst wall for the binding of lectins, and the LMS technique itself. Considering that I had no experience in LMS at all, this STSM was invaluable for me in expanding my knowledge. The LMS, together with the techniques I learned on my previous STSM, will be instrumental for processing the environmental samples for my PhD thesis, which will for the first time allow for the acquisition of data on the presence and biodiversity of protozooan parasites in surface waters in Serbia.

DESCRIPTION OF WORK CARRIED OUT DURING THE STSMS

(max.500 words)

Overall, the work I have done during the two-week visit can be divided into three sections: preparing samples for LMS, LMS itself, and *T. gondii* detection and genotyping. The latter will be performed by qPCR and RFLP techniques which are in routine use in my home laboratory; qPCR is used to amplify a 529bp repeat sequence in the genome, which affords high sensitivity and is thus suitable for monitoring the efficiency of LMS.

The LMS technique is being developed since there is no commercial IMS kit for *T. gondii* detection in water. The now routinely used IMS technique for *Cryptosporidium* and *Giardia* detection utilises the affinity of highly specific monoclonal antibodies for their (oo)cyst walls, but there are difficulties in producing sufficient amounts of specific monoclonal antibodies to *T. gondii* oocysts. This problem has led to exploring other detection methods, such as LMS which utilises lectins, of which wheat germ agglutinin (WGA) showed the best results. The main difference of lectins as compared to MAbs is their lack of specificity, allowing them to bind to most membranes, and in the case *of T. gondii* oocysts, they exclusively bind to "aged" oocyst walls. Previous research has shown that the *T. gondii* oocyst wall is different than both *Cryptosporidium* spp. and *Giardia* spp. (oo)cyst walls; the latter organisms seem to be more inert during long term storage. It has been shown that storage of *T. gondii* oocysts for more than 10 yrs seems to expose binding sites for polysaccharide molecules (such as lectins). This outcome can also be expected following the artificial ageing of oocysts, which has successfully been performed with acidified pepsin (AP) (Harito et al*.,* 2016).

LMS is based on biotinylated WGA coupled to FITC-labeled streptavidin coated magnetic bead. As beads of this type are not commercially available, the coupling and hybridization of fluorescent WGA to the beads has to be done manually. In the protocol developed in Prof. Lucy Robertson's lab, we used a solution with 400 oocysts per µl. The samples were divided into two groups. One group was treated with acidified pepsin (AP), while the second group was left untreated. We followed a protocol similar to the IMS protocol, but using WGA, and N-acetylglucosamine (GlcNAc). After LMS, the samples were observed under the fluorescent microscope.

The qPCR is used for confirmation of *T. gondii* detection by LMS, as well for monitoring LMS performance (Harito et al*.,* 2017). For the latter, we determined the difference between Ct values of the supernatant which was left after the first magnetic capture and the pellet we acquired after LMS. Also, PCR-RFLP genotyping, or even DNA sequencing can be performed following the LMS procedure.

References

Harito, J. B., Campbell, A. T., Prestrud, K. W., Dubey, J. P., & Robertson, L. J. (2016). Surface binding properties of aged and fresh (recently excreted) Toxoplasma gondii oocysts. Experimental Parasitology, 165, 88–94. https://doi.org/10.1016/j.exppara.2016.03.022

Harito, J. B., Campbell, A. T., Tysnes, K. R., Dubey, J. P., & Robertson, L. J. (2017). Lectin-magnetic separation (LMS) for isolation of Toxoplasma gondii oocysts from concentrated water samples prior to detection by microscopy or qPCR. Water Research, 114, 228–236. https://doi.org/10.1016/j.watres.2017.02.044

DESCRIPTION OF THE MAIN RESULTS OBTAINED

(max. 500 words)

LMS

LMS is done according to the following protocol. In short, biotinylated WGA coupled to FITC is attached to a streptavidin coated magnetic bead. The coupling and hybridization of fluorescent WGA to the beads is done manually. For LMS testing of spiked samples, we used tap water spiked with 400 oocysts per µl. The samples were divided into two groups. One group was treated with acidified pepsin (AP), while the second group was left untreated. We followed a protocol similar to the IMS protocol, but using WGA, and N-acetylglucosamine (GlcNAc). After LMS, the samples were observed under the fluorescent microscope.

The reason for selective treatment of groups of samples with AP was to show that binding of WGA differs, namely, untreated oocysts bind less to WGA while the AP treated ones bind more. From the untreated group very few oocysts were recovered, while significantly more were recovered from the treated group, as determined by fluorescence microscopy. One reason why such a small number of oocysts was isolated could be the nature of the oocyst wall: “younger” oocysts (untreated group) have a different wall structure which makes it difficult for WGA to bind, while “older” oocysts (treated group) showed better binding to WGA.

PCR for genotyping (done only for training purposes)

This was done by conventional nested PCR using the VeriFlex gradient module, in order to test the optimal annealing temperature. Bands, visualised on 2% agar gel using SYBRsafe dye, should gradually become more intense as the optimal annealing temperature is approached. Instead, we observed only one band at 58°C, indicating that primers for GRA6 (selected for this training exercise) are highly specific and only work at their predicted annealing temperature. The product of this PCR reaction was used as the new template for the second step. In this run, we again applied the gradient approach. This time we changed the gradient increment to 0.5°C and noticed that bands were present at all temperatures. The reason may be the narrower range of temperatures and/or the presence of high quantities of the specific GRA6 amplicon from the first run.

qPCR

The qPCR results confirmed that oocysts were indeed *T. gondii* oocysts, and it also demonstrated that the LMS procedure was properly done (without significant loss of oocysts from the spike). After 45 cycles, we saw two curves: one starting at the 25th cycle and the second at the 43rd. The first corresponds to the sample with concentrated and purified oocysts after LMS, while the second corresponds to the supernatant. The result indicated that very few oocysts were left in the supernatant and the majority captured by LMS.

FUTURE COLLABORATIONS (if applicable)

(max.500 words)

After this STSM, I feel confident that I can perform the methods for isolation, concentration and detection of *Cryptosporidium*, *Giardia* and *Toxoplasma gondii* (oo)cysts myself. Now I can begin planning for the collecting of samples for my PhD thesis.

My thesis will be based on analysis of surface water samples from different regions of Serbia, which will be sampled, and processed, and filtrates analysed by the described molecular techniques. A future collaboration with Professor Robertson's laboratory may involve molecular detection in the NMBU lab as a form of quality control of my own results (for accuracy and consistency) prior to including them in my thesis. Also, this joint work is expected to produce more ideas for new research and publications.

The results of my PhD thesis will present new information regarding protozoa in water in Serbia by offering insight into the level of contamination of water, while species typing by PCR will allow us to assess the biodiversity of waterborne pathogenic protozoa. Such data are of scientific interest *per se*, but will also be important for the healthcare system, as this information is necessary for outbreak tracing.