STSM Report: Feb 8th-Feb 19th, National Institute for Public Health and the Environment (RIVM), Bilthoven, Netherlands

I would like to express my thanks to the STSM committee for granting me the funds necessary to complete this STSM. In addition, I would like to thank the host institution (RIVM) and in particular Dr. Joke van der Giessen for the opportunity to visit her laboratory and take part in the risk ranking exercise, Dr. Marieke Opsteegh for the magnetic capture PCR training and helpful discussions and Cecile Dam for her expert instructions. And most importantly, I would like to thank them for being wonderful hosts and really making this STSM an enjoyable learning experience.

Purpose:

This STSM visit to the laboratory of Dr. Joke van der Giessen at RIVM in Bilthoven, Netherlands, consisted of a 5 day foodborne parasites risk ranking exercise and a 5 day magnetic capture PCR training. The purpose of the risk ranking exercise was to learn the underlying multicriteria based methodology and scoring principles and gain insight into the current status of foodborne parasites and their impact on public health and European economy. The purpose of the magnetic capture PCR training was to learn the technique of magnetic capture PCR, which included the sample preparation protocol, DNA capture protocol, PCR setup protocol and finally, evaluation of the results.

Description of work carried out during the STSM:

Risk ranking exercise

The risk ranking method used in this exercise is an adaptation of the method used originally by the FAO/WHO to rank foodborne parasites based on their global importance to public health. The intent of this exercise by WG1 of COST action FA1408 was to rank foodborne parasites based on their impact on pan European and regional public health and the economy. The ranking exercise was preceded by a detailed introduction and explanation of the multicriteria decision analysis methodology. Two separate criteria sets were used: 9 FAO/WHO criteria and a set of 11, which included the FAO/WHO criteria and two additional criteria selected by the organizers (designated COST) due to their perceived importance for Europe. The criteria are shown below:

Criterion	Scoring	Used by
Number of foodborne illness cases	Numerically: only cases manifesting disease	FAO/WHO and COST
Global distribution	Numerically: a) number of regions for pan European and b) number of countries for regional	FAO/WHO and COST
Acute morbidity severity	Semantically	FAO/WHO and COST
Chronic morbidity severity	Semantically	FAO/WHO and COST
Fraction of illness which is chronic	Numerically: %	FAO/WHO and COST
Case fatality ratio among illness cases	Numerically: %	FAO/WHO and COST
Likelihood of increased human burden	Numerically: %	FAO/WHO and COST
Relevance for international trade	Semantically	FAO/WHO and COST
Impact to economically vulnerable communities	Semantically	FAO/WHO and COST
Probability of introduction	Numerically: %	COST
YLD	Numerically	COST

Every participant scored each criterion first on a scale from 1 (most important) to 9 (least important) so that weights ranging from 1 to 100 could be assigned accordingly, whereby a weight value of 100 corresponded automatically to the most important criterion. These values represent the perceived importance and evaluated impact of each criterion on the final score of each parasite. Average scores and weight values obtained from polling the entire WG1 were used to calculate the final score which ultimately determines rank. WG1 was divided into groups consisting of 4-6 members representing different regions of Europe for the pan European ranking exercise, while groups for the regional ranking exercise consisted of all representatives of one region. Groups were tasked with scoring a subset of parasites for the pan European ranking or all 24 parasites for the regional ranking. Scores were assigned based on expert opinion, organizer recommended guidance literature (Hald et al 2016, Salomon et al 2015, Devleesschauwer et al 2015, Haveealar et al 2010) and other published literature with relevant data to support the scoring and then entered into Excel based scoring sheets. Each parasite was scored at least twice by different groups. Prior to finalization, each ranking graph was reviewed by the entire WG1 and differences in scoring were openly discussed.
Parasites scored in this exercise are shown below:

Anisakidae	*Entamoeba histolytica*	*Taenia saginata*
Ascaris spp.	Fasciola spp.	*Taenia solium*
Balantidium coli	*Giardia duodenalis*	Toxocara spp.
Cryptosproridium spp	Heterophyidae	*Toxoplasma gondii*
Cyclospora cayetanensis	Opisthorchiidae	Trichinella spp. other than T. spiralis
Diphyllobothrium spp.	Paragonimus spp.	*Trichinella spiralis*
Echinococcus granulosus	Sarcocystis spp.	*Trichuris trichiura*
Echinococcus multilocularis	Spirometra spp.	*Trypanosoma cruzi*

Results obtained:

Based on preliminary insight, it seems that this exercise produced a risk ranking of foodborne parasites which is similar to the FAO/WHO, despite the fact that two different criteria sets were used. Inclusion of the additional criteria, probability of introduction and YLD, had mostly a minor effect on the score for select parasites and did not change the overall ranking dramatically: *Toxoplasma gondii* congenital scored highest, while Spirometra spp. scored lowest using both sets of criteria. However, discussions revealed that the YLD criterion was exceedingly difficult to score and it was therefore decided that it be removed. Weighting will be done again on the remaining 10 criteria by each participant to generate a new set of reference values. In addition, preliminary data showed that scoring of the same parasite by different groups can differ dramatically. For instance, scores for Trichinella spp. other than spiralis showed large discrepancies between groups independent of the set of criteria used. In contrast, scoring of *Angiostrongylus cantonensis* and *Trypanosoma cruzi* showed very little discrepancy between groups. It is likely that this discordance reflects different decision making rationales: scoring based on expert opinion and scoring based on published reports. This indicates that the final ranking results have to be interpreted with caution. Discrepant scores for some parasites, even if drastic, do actually reflect the reality of the situation in the medical practice or diagnostic laboratory setting in different regions of Europe. The importance of the discrepancies as perhaps an additional source of information should therefore not be underestimated. The finalized ranking results will be published in the coming months.

Magnetic capture PCR training

Magnetic capture PCR for *Toxoplasma gondii* is a technique developed by Marieke Opsteegh in

order to improve detection of the parasite in meat samples. This method allows for the targeted capture of *T. gondii* DNA using *T. gondii* specific biotinylated capture oligos (designed to hybridize to the 529bp repeat sequence in the *T. gondii* genome) conjugated to streptavidin coated Dynabeads. The method can be broken down into three protocols: sample processing and preparation, DNA hybridization to specific oligos and capture by magnetic beads and finally real time quantitative PCR. For the purpose of this training, supermarket bought cuts of chicken, pork and beef were used. To ensure detection of *T. gondii*, a set of samples was spiked with different numbers of tachyzoites of the RH strain (25000, 2500 and 250). Initial sample preparation included cutting the meat into roughly $1cm^3$ pieces, weighing out 100g and depositing it into stomacher bags. Next, a cell lysis solution containing proteinase K was prepared and 250ml of the solution was added to each bag. 5 samples were generated from each type of meat: 1 blank sample and 4 spiked samples. To achieve tissue digestion and cell lysis, the samples were first processed by a stomacher for 120sec, then sealed and incubated in a 55°C waterbath overnight. The next day, each sample was first briefly processed in the stomacher (60sec) and then 50ml were removed and centrifuged at 3500rpm for 45min to pellet any residual meat particles. 12ml of the resulting supernatant was transferred into a clean centrifuge tube and incubated at 100°C for 10min to inactivate proteinase K. After a brief cooling period, 50µl of streptavidin-sepharose was added to each sample and the samples were incubated in a rotor at room temperature for 45min to allow for the capture of any endogenous free biotin. After centrifugation at 3500rpm for 10min to pellet the sepharose, 10ml of supernatant was transferred into a clean centrifuge tube and 10pmol of each capture oligo was added. The samples were incubated at 95°C for 15min to denature the dsDNA and allow the capture oligos access to the specific target sequences. Hybridization of the oligos was performed in a waterbath at 55°C for 45min. After the samples had cooled to room temperature, 2ml of 5M NaCl solution and 80µl of Dynabeads in BW buffer were added to each for DNA capture, which was allowed to proceed for 60min. Next, the samples were placed in a magnetic holder to adhere the Dynabeads so that the liquid could be discarded, the beads washed with 100µl BW buffer and finally, resuspended in 50µl of DNAse free water. To dissociate the specific target DNA from the Dynabeads, each sample was incubated at 100°C for 10min. The beads were again adhered to a magnet to allow for the removal of the supernatant containing the specific DNA. The supernantant was subsequently used as template for real time quantitative PCR.

Results obtained:

We were able to amplify *T. gondii* DNA in the spiked samples by real time quantitative PCR after magnetic capture, indicating that the method was performed successfully. In addition, we were able to ascertain that the method is equally successful for chicken, pork and beef without adaptation of the original protocol, suggesting that it could be directly applied to other meats. The primary advantage of this method is the ability to test up to 100g of sample in a single PCR reaction, rather than just 10-25mg which is routinely achievable with commercial DNA extraction kits. Additionally, the DNA obtained by this method is essentially free of host DNA, which can act as a PCR inhibitor. Notably, this method does not include a complete standard DNA extraction protocol employed by commercial DNA extraction kits, except for the enzymatic digestion step. Here, the components of the cell lysis buffer in combination with the high temperature incubations are sufficient to efficiently release DNA from the cells, which is then purified by hybridization to the Dynabeads. Although the lowest number of parasites used for spiking was approximately 250, we were informed that the magnetic capture method has a detection limit of approximately 130 parasites per 100g of sample.

Magnetic capture PCR is highly versatile, as specific oligos for different targets can be synthesized. In the case of *T. gondii* detection, another useful application of magnetic capture is the specific capture of DNA sequences suitable for genotyping of *T. gondii* strains. Genotyping by RFLP and microsatellite require an abundance of target DNA, which can be difficult to obtain due usually to a low parasite burden within samples. Genotyping may be additionally hampered by the presence of host DNA. Magnetic capture PCR may offer a solution to this problem. Despite the fact that a single target, such as GRA6 is less abundant than a multi-copy target such as the 529 repeat element, the specific capture of a single target suitable for genotyping should yield a higher quality sample and may thus increase the chances for successful genotyping.

While most of the steps which make up the magnetic capture method are quite simple to perform, sample preparation needs to be performed with utmost care. Sample preparation requires a significant amount of handling and manipulation which can result in contamination or cross contamination, especially when spiked controls are included in the experimental setup.

<u>Future perspectives:</u>

This STSM gave me the opportunity to meet many leading European parasitologists and learn about the impact of foodborne parasites on public health in Europe. Moreover, I have gained

invaluable insight into the challenges foodborne parasites pose to the medical community, diagnostic laboratories and authorities concerned with food safety, in terms of surveillance and gathering of reliable epidemiological data, preventing and controlling outbreaks.

After this STSM, I feel confident that the technique of magnetic capture PCR can be successfully implemented in my home laboratory. I am particularly interested in applying magnetic capture PCR to genotyping of *T. gondii* isolates. We have recently been able to isolate and genotype distinct type III strains from horse meat in Serbia after bioassays in mice. We hope that the advantages offered by magnetic capture PCR will allow us to significantly increase our capacity for genotyping and thus our insight into the strain diversity in Serbia. Dr. Opsteegh's expertise and experience with magnetic capture PCR for *T. gondii* will be an invaluable resource in that regard. Finally, I hope that this STSM has contributed to further strengthen the good partnership between the host laboratory of Dr. Joke van der Giessen and my home laboratory established through this COST action and collaboration on an EFSA project and that the result will be other joint research projects in the future.